Cell Mediated Immunity in Male Infertility

Thesis submitted in accordance with the requirements of the University of Liverpool

Doctor of Medicine Degree

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Dedicated to Dad

ABSTRACT

Although leucocytes are a normal constituent of human semen, the concentration can vary. About 20% of infertile men have leucocytopsermia (leucocytes $1x10^6$/ml) with granulocytes being the most common leucoyte present. There has always been a poorly defined correlation between the role of leucocytes and male infertility. Leucocytospermia has been shown to be associated with poor semen quality by several studies. However, other studies have stated that leucocytes and leucocyte subpopulations in semen have no role to play either in reducing the sperm fertilising capacity or in decreasing sperm quality.

Cytokine levels are significantly elevated in seminal plasma exhibiting bacterial or mycoplasma infections in the urogenital tract, but their role in male infertility is controversial. Cytokines may influence sperm motility directly or indirectly as they have been shown to negatively correlate with the number of progressively motile sperm, resulting in reduced mucosal penetration properties. Similarly, cytokines have been shown to decrease the sperm penetration capacity of zona-free hamster eggs. However, other studies have been unable to find any significant differences in the cytokine content between fertile and infertile men.

A prospective cohort study of the leucocyte subpopulations and cytokine content of seminal fluid of different categories of infertile men as well as men attending an IVF programme was undertaken. This study was aimed to identify the role of leucocytes and cytokines in male infertility. The study will help to clarify a much debated issue.

Semen samples were obtained from normal fertile donors, men being referred to the andrology and IVF clinics in Liverpool Women's Hospital and to the seminology laboratory

for routine semen analysis. Patients were placed in the followings groups namely 1) Normospermia 2) Asthenospermia 3) Oligospermia mild 4) Oligospermia severe 5) Oligoasthenospermia mild 6) Oligoasthenospermia severe 7) Azoospermia (obstructive) 8) Azoospermia (germ cell damage). These groups were determined according to the sperm count and sperm motility. These patients were also subdivided into two further groups depending on their antisperm antibody status as tested by the mixed antiglobin reaction (MAR) for IgA and IgG antibodies.

Samples were produced by masturbation after three to five days of sexual abstinence and collected in sterile containers. Swabs for bacteriological cultures including *Gonococcus*, *Trichomonas*, *Chlamydia*, *Ureaplasma*, *Mycoplasma* and general bacterial and fungal growth were taken. Leucocytes were identified using an immunohistocytochemical technique. The size of the total leucocyte population was assessed using monoclonal antibodies directed against the common leucocyte antigen. The remainder of the sample was used to identify cytokines using enzyme linked immunosorbent assay (ELISA) techniques.

Most of the studies looking into the role of leucocytes in infertility have concentrated on one or two of the leucocytes subpopulations. Similarly, the commonly reported cytokines with relation to infertility such as interleukin IL-1, IL-6, IL-8, tumour necrosis factor alpha and interferon gamma have been either studied individually or in combination. However, an extensive study to look into all the leucocyte subgroups and the various cytokines to determine their significance in male infertility and their effects on fertilisation and implantation rates in an IVF programme has not been performed. This study aimed to identify these particular leucocyte subgroups and cytokines.

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I dedicate this thesis to my father K.Seshadri who was instrumental in evoking an interest in medical research, and initiating the whole process. Unfortunately he is not around to see the fruits of the work put in.

I would also like to thank my husband without whose caring support at all stages, I could not have completed this work. My two little angels have contributed by their undemanding ways to the completion of this project. Thank you, both of you.

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
ADCC	Antibody dependent cellular cytotoxicity
AGIF	Adipogenesis inhibitory factor
APAAP	Aklaine phosphatase: antialkaline phosphatase complex
APC	Antigen presenting cell
ASA	Antisperm antibody
Anti r UreG	Anti receptor urease complex component
BSA	Bovine serum albumin
BTB	Blood testis barrier
C	Complement
CD	Cluster of differentiation
CMI	Cell mediated immunity
COX-2	Cyclooxygenase
CTL	Cytotoxic T lymphocyte
CXCR1&2	Chemokine receptor
DC	Dendritic cells
ET	Embryo transfer
FAS	Fluorescent antibody stain
FasL	Fas ligand
FC	Fragment crystallisable
FSH	Follicle stimulating hormone
γs lymphocytes	Gamma lymphocytes
GIFT	Gamete intrafallopian transfer
GM-CSF	Granulocyte macrophage colony stimulating factor

GnRH	Gonadotrophin releasing hormone
HOS	Hypo osmotic swelling
HPF	High powered field
Hsp	Heat shock protein
ICSI	Intra cytoplasmic sperm injection
IFN γ	Interferon gamma
IFN	Interferon beta
IkB	Inhibitory kappa B protein
IL	Interleukin
IL-R	Interleukin receptor
IL-RA	Interleukin receptor antagonist
IUI	Intrauterine insemination
IVF	*In vitro* fertilisation
IVF-ET	*In vitro* fertilisation- embryo transfer
JAK	Janus kinase pathway
LIF	Leukemia inhibitory factor
LGL	Large granular lymphocyte
LPS	Lipopolysaccharide
LIF	Leukaemia inhibitory factor
LH	Luteinising hormone
LREC	Local research ethics committee
MAGI	Male accessory gland infection
MAPK	Mitogen activated protein kinase
MC5b-9	Membrane bound terminal complement complex
MC	Complement membrane attack complexes

MHC	Major histocompatibility complex
MMPS	Matrix metalloproteases
mRNA	Messenger ribo nucleic acid
NASP	Nuclear autoantigenic sperm protein
NF-Kb	Transcription factor kappa B protein
NHS	Normal human serum
NK	Natural Killer
NKkB	Natural killer kappa B
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PGE2	Prostaglandin E
PMN	Polymorphonuclear
ROS	Reactive oxygen species
RPM	Revolutions per minute
SCF	Stem cell factor
SMT	Sperm migration test
SpAbs	Sperm antibodies
SPSS	Statistical package for social sciences
STAT	Signal transducers and activation of transcription pathway
TATA	Sequence of nucleotides
TBS	Tris buffered saline
TCE	Tumour necrosis factor converting enzyme
TCR	T cell receptors
TGF	Transforming growth factor
Th	T helper cells

TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
UreG	Urease complex component UreG

DECLARATION

I certify that the work contained in this thesis is all my own. It has not been submitted in whole or part for consideration for another degree or diploma. All studies contained within this thesis have received ethical approval from the Local Research and Ethics Committee.

October 2011 **Srividya Seshadri**

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CHAPTER 1: MALE INFERTILITY

1.1 Infertility

Infertility has been defined as a failure to conceive after a year of unprotected intercourse with the same partner. Use of this time period, while arbitrary, was based upon a study of 5574 British and American women engaging in unprotected intercourse who ultimately conceived between 1946 and 1956. Among these women, 50 percent conceived within three months, 72 percent within six months, and 85 percent within 12 months[1]. If a pregnancy does not occur by three years, persistent infertility is very likely in the absence of medical intervention[2]. Studies indicate that one in six couples around the world have difficulties in conceiving at some point in their lives, adding up to 50-80 million people[3]. The available information indicates that about 15% of all couples in the western world are infertile[4].

Infertility depends on both male and female factors, as more than one factor may coexist in both partners. In the past decade the WHO has indicated that a male cause may be present in about 43% of infertile couples[5]. Recent epidemiological studies in the UK have indicated that abnormal semen[6] or defective sperm function[7] may be the most common single defined cause of infertility in approximately 25% of couples investigated. Historically, the workup for the infertile couple focused primarily on the condition of the female. However, the birth of Louise Brown, the first successful *in vitro* fertilisation, heralded a major step forward in dealing with one of the causes of infertility: tubal infertility[8]. This has motivated interest in assisted reproduction techniques such as controlled ovarian stimulation, intra-cytoplasmic sperm injection to help overcome male factor infertility and many others. Thus, assisted

reproduction technology has allowed a better understanding of the process of human embryo development and has opened up avenues of research into the causes and cures of infertility. The trend has now shifted, such that the male partner in an infertile liaison may be investigated first. If an abnormality is detected in the seminal analyses, the couple can then be referred to a clinical andrologist rather than a gynaecological infertility clinic to avoid unnecessary female investigations.

Male infertility, internationally, has been noted to vary, depending on various factors such as social habits, environmental conditions and genetics. However, recent studies have demonstrated that there is poorer semen quality, reduced sperm numbers and reduction of fertility in men today as compared to that of fifty years ago[9-11].

1.2 Male Infertility

Studies cited earlier have indicated that the male factor accounts for approximately 30% of the cases of infertility[12]. Many factors can interfere with the process of sperm production. It could be deficiencies in the main hormones leading to sperm production classified as pre-testicular infertility. Testicular infertility could include factors damaging the sperm producing cells. Mechanical blockage of the outlet system for ejaculation and failure of erection and ejaculation is termed as post-testicular infertility. Table 1.1 summarises the distribution of the main aetiological causes of male infertility.

The unexplained forms of male infertility may be caused by several factors such as chronic stress, endocrine disruption due to environmental pollution, reactive oxygen species and genetic abnormalities. Table 1.2 gives the standard values for semen analysis according to the 1999 WHO criteria[13].

Table 1.1: Aetiology and distribution (%) of male infertility among 7057 men. Adapted from guidelines on male infertility 2005[14].

Aetiology	Distribution (%)
Sexual factors	1.7
Urogenital infection	6.6
Congenital anomalies	2.1
Acquired factors	2.6
Varicocele	12.3
Endocrine disturbances	0.6
Immunological factors	3.1
Other abnormalities	3.0
Idiopathic abnormal semen (OAT syndrome) or no demonstrable cause	68.0

Table 1.2: Overview of standard values for semen analysis according to the 1999 WHO criteria[13].

Parameters	Values
Volume	≥ 2ml
pH	7.0-8.0
Sperm concentration	≥ 20 million/ml
Total number of spermatozoa	≥40 million/ejaculate
Motility	≥ 50% with progressive motility or 25% with rapid motility within 60 mins after ejaculation
Morphology*	≥ 14% of normal shape or form
Viability	>50% of spermatozoa
Leucocytes	<1 million/ml
Immunobead test	<50% spermatozoa with adherent particles
MAR test**	<50% spermatozoa with adherent particles

*Assessment according to Kruger and Menkfeld criteria.
**MAR= Mixed antiglobulin reaction.

WHO revised these criteria in 2010. In accordance to the new WHO criteria, over 15 million sperm per millilitre is considered to be normal for sperm count, 40% for motility and 4% or more of the observed sperm have normal morphology[15]. If the semen analysis is initially normal according to the mentioned WHO criteria, one test should be sufficient. However, further andrological investigation is only indicated if the results are abnormal in at least two tests. There are several causes of male infertility. The causes of male infertility are elaborated as shown in Table 1.3.

1.3 Immunological Factors in Infertility

A recently recognised class of infertility is immunological infertility, most often characterised by the presence of antisperm antibodies (ASA) in one or both partners (semen, blood, cervical mucous or follicular fluid).

1.3.1 Antisperm Antibodies

The presence of antisperm antibodies in infertile men was first reported in 1954[17]. The incidence of sperm autoimmunity in infertile couples is 9-36% in contrast to 0.9-4% in the fertile population. Males may resorb senescent sperm and females may adsorb sperm from coitus, thereby producing low titres of sperm antibodies seen in fertile couples[18].

The incidence of detection of sperm antibodies in the infertile male is up to 21% and in the female up to 23%[19]. An immunological aspect may contribute to 5-15% of male infertility causes. Antisperm antibody (ASA) can be defined as immunoglobulins of the IgG, IgA and / or IgM isotype that are directed to various aspects of the spermatozoa (head, tail, midpiece or combination thereof).

Table 1.3: Causes of male infertility. Adapted with permission from British Journal of Hospital Medicine [16].

Primary testicular failure

Congenital disorders
Klinefelter's syndrome (XXY) and its variants (XXY/XY; XXXY)
Cryptorchidism
Myotonic dystrophyFunctional prepubertal castrate syndrome (congenital anorchia)
Varicocele (congenital weakness of the mesenchyme of the blood vessels)
Androgen insensitivity syndromes
5-alpha-reductase deficiency
Y chromosome deletions

Acquired disorders
Viral orchitis (mumps, echovirus, arbovirus)
Granulomatous orchitis (leprosy, tuberculosis)
Epididymo-orchitis (gonorrhea, chlamydia)
Drugs (e.g., alkylating agents, alcohol, marijuana, antiandrogens, ketoconazole, spironolactone, histamine receptor antagonists)
Ionizing radiation
Environmental toxins (e.g., dibromochloropropane, carbon disulfide, cadmium, lead, mercury, environmental estrogens and phytoestrogens)
Hyperthermia
Immunologic disorders, including polyglandular autoimmune disease
Trauma
Torsion
Castration
Systemic illness (e.g. renal failure, hepatic cirrhosis, cancer, sickle cell disease, amyloidosis, vasculitis, celiac disease)

Secondary testicular failure (Hypothalamic-pituitary disorders)

Congenital disorders
Congenital GnRH deficiency (Kallmann syndrome)
Hemochromatosis
Multiorgan genetic disorders (Prader-Willi syndrome, Laurence-Moon-Beidl syndrome, familial cerebral ataxia)

Acquired disorders
Pituitary and hypothalamic tumors (macroadenoma, craniopharyngioma)
Infiltrative disorders (sarcoidosis, histiocytosis, tuberculosis, fungal infections)
Trauma, postsurgery, postirradiation
Vascular (infarction, aneurysm)
Hormonal (hyperprolactinemia, androgen excess, estrogen excess, cortisol excess)
Drugs (opioids and psychotropic drugs, GnRH agonists or antagonists)

Systemic disorders
Chronic illnesses
Nutritional deficiencies
Obesity

Extra testicular disturbances

Epididymal dysfunction
Radiation
Drugs
Infection- Mumps, Mononucleosis, Hepatitis, TB, Gonorrhoea, Mycoplasma, Candida, Trichomonas, Reiters)
Young's syndrome (functional obstruction at the level of the caput epididymis)

Abnormalities of the vas deferens
Congenital absence
Infection
Vasectomy

Ejaculatory dysfunction
Cerebral and spinal cord disease
Autonomic dysfunction- drugs, diabetes and surgery
Premature ejaculation, retrograde ejaculation and anorgasmy,
Spinal and peripheral nerve injuries

Idiopathic

In other words, it is an immunoglobulin with antibody activity against the sperm antigen that plays a role in infertility. However, not every antibody that binds to the sperm surface influences sperm function[20]. Immunoglobulin M is too large a molecule to cross into the semen from the systemic circulation. ASA concentration may be of importance in affecting sperm function. Hence there are some protective mechanisms against the development of ASAs and the most important amongst them is the blood testis barrier to which the immune privilege of the testis was originally attributed to. Figure 1.1 describes the blood testis barrier.

Protective mechanisms preventing ASA production - The blood-testis barrier (BTB) and the tight junctions between Sertoli cells appear to play a major role in keeping the developing spermatozoa and immune system separate. It prevents those testicular cells expressing "foreign" antigens from coming into contact with lymphoid tissue and immunocompetent cells from entering the seminiferous tubules. This creates favourable conditions for spermatogenesis and sperm survival in the testicular fluid, and sperm maturation in the epididymal fluid. It also prevents the occurrence of autoimmunity after puberty. Therefore, alteration of the blood-testis barrier and the epididymal blood-epithelium barrier allows the production of ASAs and, hence, may lead to infertility. The BTB is commonly breached by physiological leakage of normally sequestered sperm antigens. Also the tight junctions do not protect all intratesticular sperm autoantigens from the immune system that lie below the tight junctions in the basal compartment of the seminiferous tubule. Recent research has shown a previously unknown level of complexity with a multitude of factors both physical and immunological necessary for the establishment and maintenance of immunotolerance in the testis[22].

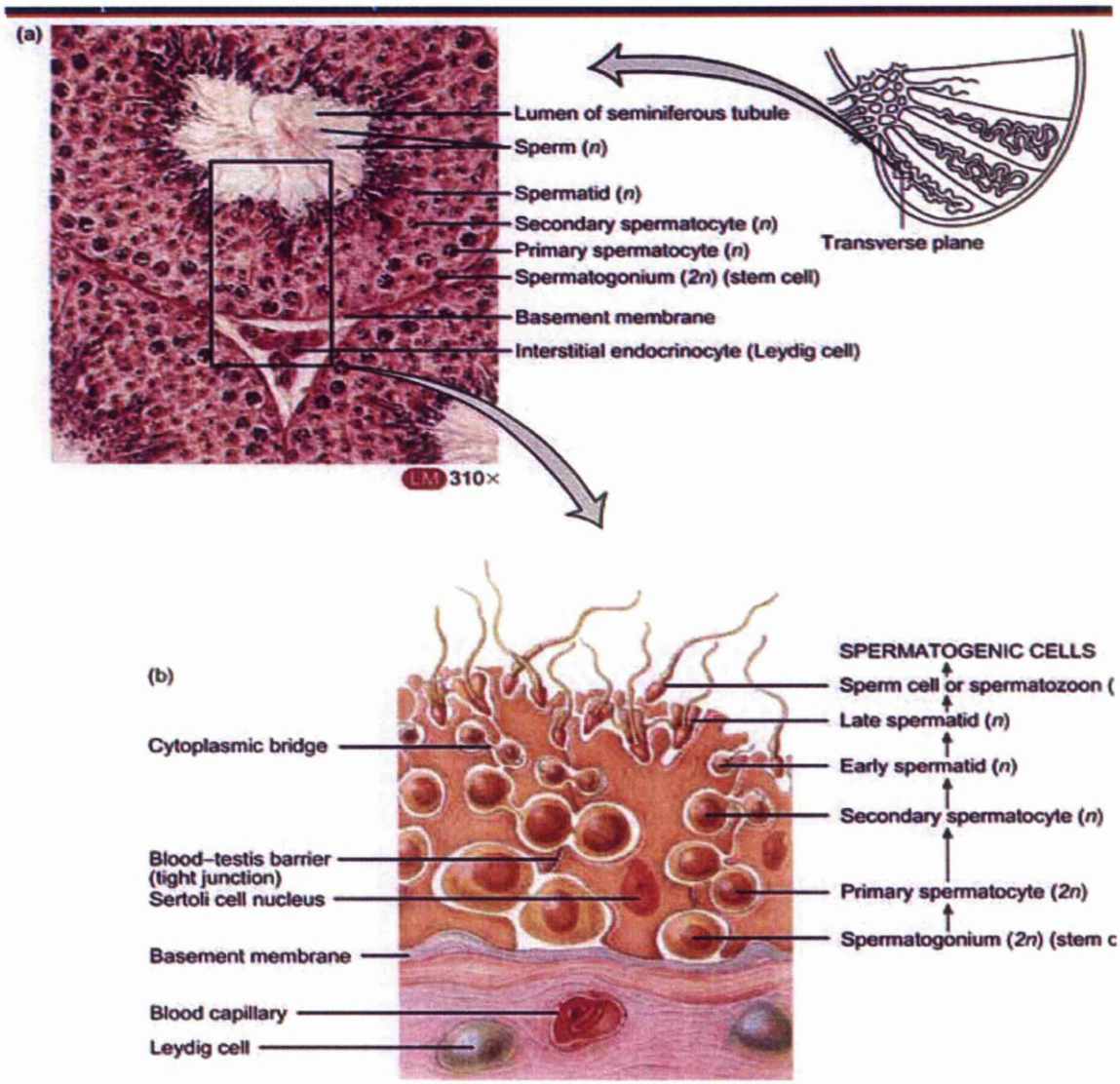


Figure 1.1: Microscopic anatomy of the seminiferous tubules. (a) Transverse section of several seminiferous tubules. The blood capillaries are within the interstitium. (b) Transverse section of a portion of a seminiferous tubule. The blood-testis barrier is located in the seminiferous tubule between the Sertoli cells. Reprinted from Tortora and Grabowski (editors) with permission from John Wiley & Sons, Inc [21].

Besides the blood testis barrier and the diminished capability of the large testicular resident macrophage population to mount an inflammatory response, it is the constitutive expression of antiinflammatory cytokines in the testis that represents an essential element for local immunosuppression.

Other protective mechanisms besides the blood-testis barrier and the blood-epithelium barrier have also been identified. Immunosuppressive substances have been found in semen and follicular fluid. Moreover, T lymphocytes in the human immune system, which partially mediate the normal state of immunologic unresponsiveness toward sperm autoantigens through cytokines, also play an important role in preventing the autoimmune response. Components of seminal plasma and polymorphonuclear neutrophils in semen could eliminate nonviable sperm or debris[23].

Some of the proposed mechanisms of immune protection are:

1. Immunologic tolerance induced by low grade "leakage" of sperm antigens;
2. Immunomodulatory mechanisms within testis e.g. steroids, macrophages, suppressor cells which may prevent activation of immune response; and
3. Immunomodulation distal to the testis (T lymphocytes mediated cytokine production in the epididymis and immunosuppressive activity of seminal plasma).

Crude sperm protein preparations are highly immunogenic in all species. There is considerable evidence that under normal circumstances some interaction occurs between sperm and the immune system within both the testis and epididymis and that immunomodulatory mechanisms act to prevent SpAb formation. There is also clear evidence for the presence of immunosuppressive cytokines in the testis and seminal fluid[24;25]. The formation of SpAb is influenced by a number of factors including:

1. flushing of sperm from the genital tract;
2. degradation of sperm antigens by extra-cellular enzymes;
3. phagocytosis of sperm by somatic cells and macrophages;
4. immunopotentiation by other foreign antigens in the genital tract;
5. paternal lymphocytes present in semen; and
6. seminal immuno-suppressive factors.

SpAbs are rare in fertile couples implying, but not proving a role for SpAbs in infertility[24;26].

1.3.2 Factors leading to ASA production

Chronic Infection: Antisperm antibodies were found in 25-56% of men with chronic prostatitis[27]. It has been suggested that the female's isoimmunity to sperm is associated with sperm autoimmunity in their husbands[18]. Women with pelvic infection have a higher incidence of sperm immunity (up to 59%). Antibody production is linked to *Chlamydia trachomatis*, *Mycoplasma species* and *Ureaplasma urealyticum* infections. It has been reported that Heat-shock protein (Hsp) 60 in human seminal fluid was associated with the formation of ASAs[28]. Hsp 60 is associated with a humoral immune response to *C. trachomatis* or other infection agents, indicating that genital infection could be an important factor causing ASA production[28].

The possible relationship between ASA production and *Ureaplasma urealyticum* infection was also investigated. It was confirmed that *U. urealyticum* and human sperm membrane proteins share cross-reactive antigens (61, 50 and 25 kDa)[29]. Among the cross-reactive antigens, the urease complex component UreG of *U. urealyticum* was discovered. Furthermore, the cross-reaction between human NASP (nuclear autoantigenic sperm protein) and UreG was verified. Both anti-rUreG antibodies and antiserum against synthetic peptide NASP393-408 inhibited mouse sperm-egg binding and fusion. After immunisation with

rUreG or synthetic peptide, 81.2% and 75% of female mice became sterile, respectively[29]. These findings proved that the cross-reactive antigens shared in sperm and microorganisms induce ASA production and infertility.

Vasectomy and Vasovasostomy: More than 50-70% of men develop ASAs after a vasectomy, and there is limited success in the regaining of fertility, even after successful surgical reanastomosis by vasovasostomy. This has been attributed to the presence of ASAs[30]. The highest incidence of titres is one year after vasectomy, but titres can be found as early as six months or as late as 20 years post-operatively. These ASAs are causative factors in infertility, because disappearance of ASAs causes return of fertility. However, considerable disagreement remains as to whether antibodies are the primary causative agent. No significant changes were observed in the prevalence of the antibodies over the period following vasectomy, and in patients with and without postoperative sperm granulomas[31]. Sperm antigens are in abundant supply in vasectomised men as a result of the continuous resorption of spermatozoa after vasectomy. Therefore, it is possible that undetectable antibody titres reflect high levels of ASAs circulating as immune complexes.

Other factors: Heavy metals can also adversely influence reproduction since in sensitive individuals they are able to alter the immune responses including autoantibody production, which can then cause infertility[32]. For example, after mercury stimulation, less IFN-γ and more ASAs were produced by the lymphocytes of patients[33]. This suggested that the release of metal ions from dental materials can be one of the stimulating factors that may adversely affect fertility. Testicular cancer and testicular torsion patients also have a markedly high incidence of ASAs[34]. The former may be related to radiation therapy and severe damage of seminiferous tubules. Torsion causes a breakdown of the blood testis barrier (sympathetic orchiopathia) and studies in rats have shown the production of ASA after torsion[35]. In

addition, a high ASA rate (43.1%) was observed among prostitutes[36]. It may be related to repeated inoculations with multiple sperm antigens and / or microorganisms.

The possible effects of immunologic reaction to fertility are:

1. Disordered spermatogenesis resulting in oligospermia and azoospermia;
2. Binding of antibodies to post testicular spermatozoa and inhibiting their effective transport in the male reproductive tract;
3. Auto agglutination of ejaculated spermatozoa;
4. Sperm cytotoxicity mediated by sperm antibodies;
5. Direct immobilising effect of sperm antibodies on spermatozoa in the female tract;
6. Enhancement of phagocytic clearance of spermatozoa by macrophages;
7. Inadequate spermatozoal penetration of cervical mucus;
8. Disorders of sperm capacitation and acrosome reaction;
9. Blockage of sperm-ovum interaction;
10. Induction of sperm immunity in the female; and
11. Post fertilisation reproductive failure and occult abortion.

1.3.3 Role of Antisperm antibodies in infertility

Antibodies directed towards various sperm antigens can result in reduced fertility in men. Immunological infertility is probable if more than 50% sperm are bound to IgG or IgA antibodies[37]. As the level of antibodies increases, sperm function can be impaired, and the chance of spontaneous pregnancy declines. Results from several large studies on the presence of sperm agglutinins in fertile and infertile men indicate a high correlation between sperm agglutinins and complement dependent immobilising antibodies. ASAs can bind to antigens on sperm membranes, but subcellular structures cannot be reached by ASAs in living cells. Therefore, it is speculated that the function of transmembrane proteins may be altered by the

ASAs concerned. Another possible explanation is via complement-mediated membrane damage. ASAs could inactivate human sperm motility in the presence of complement, showing that complement-dependent inactivation of sperm motility might be the biological mechanism of female infertility[38].

IgG- and C3-bound motile sperm are adhered to human neutrophils *in vitro* at the acrosomal region of the sperm head. This adhesion potentiates the localised release of oxygen radicals along with activated C3 fragments, leading to sperm-motility loss[39] as high as (43-87%) and ultimately leading to sperm lysis[40]. CD11b / CD18 glycoprotein complex (CR3) have also been implicated in ASA- and complement-mediated immune destruction of motile sperm by neutrophils[41]. A receptor on the sperm surface has been identified which increases its susceptibility to immobilisation[42]. Evidence exists that sperm reactive autoantibodies may impair sperm penetration into cervical mucus from seminal plasma. This can be shown both clinically at postcoital testing and *in vitro*. Spermatozoa obtained from known fertile donors and previously shown to be able to penetrate human cervical mucus have failed to do so following incubation with antibodies *in vitro*[43].

The presence of ASAs in cervical secretions is not frequent but nevertheless when present it is a severe cause of infertility[44]. In the genital tract, IgA has been observed to be primarily present in semen, cervical mucus or follicular fluid, since the production of IgA is closely associated with mucosal immunity. It was reported that infertile men had more IgA and other secretory components in semen than controls, and that IgA was considered responsible for the reduced penetration of spermatozoa into cervical mucus and for the 'shaking phenomenon' (jittering *in situ*) seen when sperm actually enter the mucus[45]. Impairment of sperm penetration into the cervical mucus appears to be of consequence in the following mechanisms: the activation of the complement cascade by immunoglobulins attached to the

sperm surface, at the end of which cell lysis and initiation of the phagocytic process of the sperm may take place. The complement induced cell lysis in the female reproductive tract depends on the immunoglobulin class of the antibody. IgM is far more effective than IgG, while some IgA subclasses are unable to interact with the early complement components. During their residence in the cervical mucus, sperm are exposed to complement activity. The complement activity in cervical mucus is approximately 12% of that of serum[46]. Thus, it may take longer for sperm immobilisation to occur. There appears to be some reaction between the cervical mucus and the Fc region of the antibody causing binding of the sperm to the cervical mucus[47]. This process could contribute to the binding of spermatozoa to the antibody and the immobilisation of this complex by the cervical mucus that is seen in immunological infertility.

Antibody-coated sperm may activate lymphocytes, thus leading to the production of ASAs. This may be an additional mechanism that leads to infertility. Mononuclear cells, including lymphocytes, monocytes and macrophages, may play a role in infertility caused by ASAs. Cytokines secreted by these mononuclear cells may also have roles; however, these are unclear at present. It has been reported in one study that no significant difference was found in the concentration of leucocytes or subpopulations of these cells (monocytes, lymphocytes and granulocytes) between fertile, sterile without ASAs and immunological sterile groups[48]. It is possible that both the presence of ASAs and the increase of leucocytes in semen are manifestations of an immunological response or a common etiological factor of genital tract infections, and they are not interrelated. In contrast, a significant increase in large granular lymphocytes (LGL) was detected in semen samples from immunological sterile patients when compared with fertile ejaculates and sterile ejaculates without ASAs[48].

The LGLs form the population responsible for antibody-dependent cellular cytotoxicity (ADCC). An expansion of these cells is reflected by an increase in ADCC effector cells. Immunoregulation by ADCC induces the disappearance of the antigens recognised by each antibody. Thus, the appearance of ASAs in an ejaculate could generate the expansion and activation of LGLs to decrease ASA production by B lymphocytes. Nevertheless, LGL activity may be a specific cellular mechanism operative in limiting the fertilising ability of these spermatozoa. Antisperm cell-mediated immunity (CMI) has been associated with infertility in men and women. Sperm antigens can specifically induce CMI factors that have detrimental effects on sperm motility and preimplantation embryos. Despite the observation of antisperm CMI responses in all individuals of the following groups: infertile men and women, fertile women, virgin women, oligozoospermic men, and men with andrological disorders, the role of antisperm CMI in reproductive processes is still unclear.

Fertilisation is significantly reduced ($p<0.001$) only if both IgA and IgG antibodies are present in semen but there is no reduction if either class is present alone[49]. High levels (>80%) of ASA bound sperm were less likely to fertilise. In patients with low levels of antibodies, ASA-free sperm might contribute to higher fertilisation rates in IVF[50]. The presence of both IgG and IgA in the seminal plasma was associated with poor post coital test results and a reduced rate of fertilisation of human oocytes[51]. Lower fertilisation rates and a lower number of transferred embryos were found in couples with ASA on the spermatozoa of couples undergoing IVF when IgG was the major immunoglobulin involved[52].

Antisperm antibodies can also impair the fertilisation process at the level of the acrosome reaction, of the zona pellucida recognition and penetration, and of the sperm vitellus interaction[53]. ASA binding to the sperm head influences the acrosome reaction[54]. A number of spontaneously occurring ASAs were shown to enhance the number of acrosome reacted

sperm[55]. In addition, there is evidence that some surface bound antibodies may not interfere with fertilisation but with subsequent embryo cleavage of fertilised ova[56]. It was reported that the fertilisation rate in the antisperm antibody group (61.3%) was significantly lower than that in the control group (76.8%)[57]. Although sperm-immobilising antibodies prevent sperm-egg interactions, they do not seem to have any adverse effects on the likelihood of pregnancy[57]. Interestingly, active immunisation with many of these molecules does not inhibit fertility *in vivo*[58]. Moreover, the occurrence of a cellular or humoral immune response against sperm seems to augment the uterine receptivity for the implantation of fertilised ova or blastocyst, and to be favourable for a successful pregnancy by the IVF-embryo transfer (ET) procedure[59].

In spite of the difficulty to explain these contradictory results, it was generally accepted that most autoantibodies occurring in biological fluids do not cause autoimmune diseases[54]. It has been reported that there was no statistically significant difference in the pregnancy rate between those women with and without ASAs[60]. These conflicting studies about the effects of ASAs on fertilisation could be due to inadequate experimental approaches in evaluating antisperm immunity [61].

1.3.4 Indications for antisperm antibody testing

The indications of antibody testing are:

1) Abnormal semen analysis- Clumping / agglutination, low motility, poor sperm viability and shaking of sperm in cervical mucus rather than proper motility;
2) Abnormal postcoital test- Low number of sperm in mucus, poor motility and shaking in place of motility; and
3) Identifiable risk factors such as:
    - Abnormal *in vitro* cervical mucus penetration tests;

- Abnormal sperm penetration assay;
- Failed or low fertilisation in IVF;
- Unexplained infertility after couple's evaluation.

Methods of ASA Testing- These are the commonly used methods to test for ASA[62].

1) Sperm agglutination tests;
2) Sperm immobilisation test;
3) Immunobead test and mixed antiglobulin reaction;
4) Immunofluorescence assay (very inaccurate);
5) Enzyme-linked immunosorbent assay (very inaccurate);
6) Radiolabeled antiglobulin assay;
7) Flow cytometry;
8) Immunoblotting;
9) Affinity chromatography.

The ASAs detected by different methods are not identical. Comparative investigations using various assays to detect levels of antibodies to human spermatozoa have produced widely varying results. A possible explanation for the observed wide variation in results from different methods to detect ASAs may be the different target antigens used in assays. Although there is reason to accept antibody-mediated antisperm immunity as a cause for subfertility, the routinely used methods are not reliable for the detection of specific ASAs. The methods described so far have been hindered by co-detection of undesired non-specifically bound antibodies.

1.3.5 Treatment Modalities

There have been a wide range of treatments proposed for ASA:

1. Condom therapy- Is no longer advised as it was once thought that decreasing the antigenic load may be beneficial in the infertile female. However, it is likely that the antibody titres will rise once the therapy has been stopped.
2. Immunosuppressive therapy- The method of immunosupression most commonly used is steroid therapy. Different regimes have been used in the past with varying results. Low dose continuous therapy using 5mg of oral prednisolone for three to twelve months improved the sperm count and a pregnancy rate of 14% was achieved. However, the sperm antibody titres remained significantly high[63]. Steroids suppress macrophages and T cells and thus inhibit antibody production by B cells and proliferation of B cells clones. It also suppresses proinflammatory cytokine production by down regulating the T-helper-type-1 cells (Th1)[64]. Vitamin D3 acts by inhibiting monocyte derived cytokines and T cell activation. Combined treatment regime of Vitamin D3 with dexamethasone has shown not alone to improve sperm motility and viability but also to decrease ASA levels[65]. Most of the steroid studies are not controlled trials and hence makes it difficult to evaluate outcomes and come to a meaningful conclusion whether steroid therapy is effective in immunoinfertility.
3. Assisted reproduction- There are three important assisted reproduction techniques.

   IUI (intrauterine insemination) - Intrauterine insemination has been found to be useful for treatment of female and male immunoinfertility. Sperm passage through the female genital tract and the cervical canal is facilitated by IUI. Washing of the sperm may also help in removing some of the IgG bound to the sperm surface[66].

GIFT (gamete intrafallopian transfer) - GIFT was able to achieve 43% pregnancy rates per couple and 24% per cycle in patients with immunoinfertility[67].

IVF and ICSI (intracytoplasmic sperm injection) - IVF can help in cases of female immunoinfertility but not male immunoinfertility as albumin is used instead of the female partner's serum in the insemination medium. ICSI was able to achieve almost equal fertilisation rates and cleavage rates in the ASA+ve group compared to the ASA-ve group. However, there was increased pregnancy loss in the immunoinfertile group[68].

4. Semen manipulation - Different techniques have been tried to either decrease the binding of ASA to sperm or remove the bound ASA from sperm[69]. However, the various techniques mentioned such as sperm washing, swim-up, dilution, cryopreservation, immunomagentic sperm separation, immunobead incubation and proteolytic enzyme treatment etc. have had only limited success.
5. Fertilisation antigen-1 treatment - Immunoadsorptiontechnique using humansperm FA-1 antigen would help remove autoantibodies from the surface of sperm cells of ASA+ve men and thus increase their fertilising capacity. A study has shown that sperm motility and acrosome reaction rates were improved by this technique and this treatment did not have any deleterious effect on the foetus[70].
6. Soluble complement receptor treatment may be beneficial in treating complement mediated sperm damage[39].
7. Fab fragments treatment - Studies in rabbits have been suggested that *in vitro* treatment of sperm with Fab fragments may help to protect it from immune attack[71] *in vivo*.

8. Chymotrypsin treatment (incubation of sperm with chymotrypsin) appeared to improve fecunditiy rates when compared with non treatment[72].
9. Plasmapheresis and hemosorption - A study has shown that this technique helped to improve sperm parameters and subsequent ART and pregnancy outcomes by decreasing ASA levels and normalising humoral and cellular immunity[73].
10. Miscellaneous - Acupuncture, Chinese herbal treatment, monoclonal antibody therapy and zinc therapy have also been tried with varying success[74-76].

Despite various treatment modalities being available with regards to male immunoinfertility, most of them have met with only limited success.

1.4 Infection

It has long been appreciated that chronic inflammation and systemic infection are associated with decreased reproductive capacity. Infections account for almost 15% of cases of male infertility seen in infertility clinics[77]. Inflammatory diseases have been established as hazards to male reproductive function and fertility. General acute and chronic infections are associated with disorders in steroidogenesis and spermatogenesis resulting in temporary or permanent male infertility[77]. Urethritis, prostatitis, orchitis and epididymitis have been described as male accessory gland infections (MAGI) by the WHO.

Infections of the male accessory glands have been considered as causes of male infertility for decades. MAGI appear to affect reproductive function as indicated by decreased number, density, and motility of spermatozoa and alterations in seminal plasma markers[78-80]. Different pathophysiological concepts have been established describing the effects of MAGI on the reproductive system, and spermatozoa respectively.

These concepts involve direct effects exerted by pathogenic microorganisms in the testis, accessory glands, and spermatozoa[81], alterations in male accessory gland function due to general or local infections[59;78] and immunological events resulting from the immune defense of the body against invading microorganisms[82]. However, substantive data is lacking to confirm that these diseases have a negative influence on sperm quality[83].

Nevertheless, infections of the male genito-urinary tract may contribute to infertility by adversely affecting sperm function, causing anatomical obstruction to the efferent ducts of the testis or initiating a leucocyte response. The majority of infertile males, due to infection, are asymptomatic. However, an elevated seminal leucocyte count, pyospermia, can be associated with male infertility and may reflect an infectious or inflammatory disorder and can present with urinary symptoms and even epididymal and testicular pain[84].

The current literature defining the role of infection in male infertility is inconsistent and the mechanism by which seminal leucocytes and microorganisms may impair fertility remains controversial. Amongst many studies conducted in this field, one study has reported increased total leucocyte, granulocyte, monocyte and CD8+ T cell levels in men with infertility and pyospermia, causing an adverse effect on sperm concentration, velocity and motility[82].

Another study has reported that the strongest correlation with an abnormal sperm penetration assay was the increased presence of leucocytes in semen, but here too no correlation between semen bacterial culture results and sperm penetration scores was noted[85]. Two other studies have found that the semen parameters that correlated most significantly with a reduced ability to fertilise oocytes were a slow rate of forward progression of sperm and an elevated number of leucocytes in semen [86;87].

Macrophage activation occurs most effectively by the combination of exposure to microbial products (through the receptors) and cytokines particularly IFN-γ. The complete activation of the macrophages, including the ability to kill intracellular microbes, is helped by IFN-γ. IFN-γ is so potent becauses it enhances several microbicidal pathways, including both the respiratory burst and the generation of nitric oxide.

Figure 1.2 shows an overview of T cell mediated immunity to bacteria and fungi. The Th1T cells provide both IFN-γ and B cell help to produce IgG subclasses for opsonization of bacteria. $CD8^+$ cytotoxic T lymphocytes (CTL) can release intracellular organisms by killing the infected cell. Even macrophages may be targets of CTLs because when infected with intracellular bacteria they can become refractory to activation and less able to kill the organism themselves.

CD8 T cells are activated by peptides presented via MHC class molecules, primarily derived from microorganisms that reside in the cytoplasm, such as viruses and some intracellular bacteria that escape the phagosome such as *Listeria species*. Other pathogens that do not escape the phagosome (such as *Mycobacterium tuberculosis*) can still induce CD8 T cell responses via cross-priming, in which infected and apoptotic host cells release antigenic fragments that are taken up by dendritic cells (DCs).

Figure 1.4 shows that the effector CD8 T cells (CTL) provide protection by releasing proinflammatory and macrophage-activating cytokines and killing infected host cells via perforin release and Fas. In some cases, the release of granulysin from the CTL can also result in killing of the pathogen. As we can see from Figure 1.3, chemokines like TNF are released which cause an increase in the inflammatory cell recruitment.

Immunological events include the migration of white blood cells to the site of infection due to chemotaxis and the secretion of cytokines that are necessary for the communication between immune-competent cells. Cytokines appear to be responsible for most pathophysiological events associated with infection and are the decisive factors that determine the pathology of an infectious disease.

Several early reports have suggested a relationship between a genital infection and the production of sperm autoantibodies[88]. Furthermore, a sexually transmitted disease also appears to predispose men to the development of sperm antibodies[89]. Other studies have shown that in non-symptomatic men under fertility investigation, a previous history of prostatitis or urethritis was associated with a marked increase in the incidence of sperm antibodies[90].

Circumstantial evidence points towards chronic inflammatory conditions in the male reproductive tract as a major cause of disturbances in sperm quality. However, a greater acknowledgement of its contribution to male infertility needs to be ascertained. Hence screening for male genital tract infections is important.

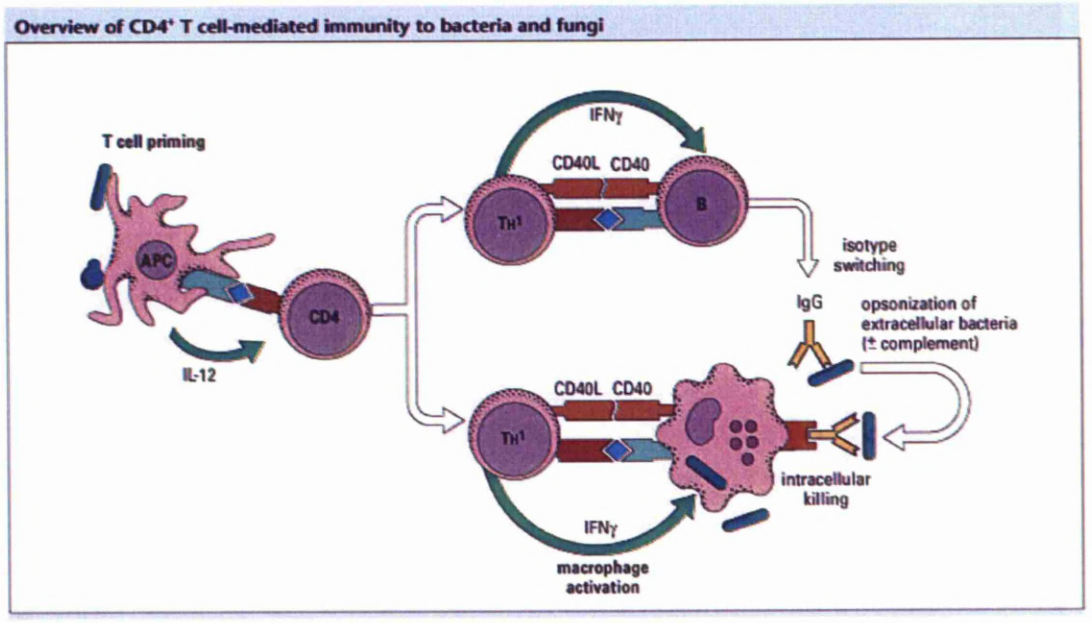


Figure 1.2: Optimal activation of macrophages is dependent on Th1 CD4 T cells. The most important source of IFN-γ during adaptive immune response to intracellular bacteria is from Th1 CD4$^+$ T cells[91]. Adapted from Immunology, Male D, Brostoff J, Roth D, and Roitt I (editors), with permission from Elsevier Ltd, 2006.

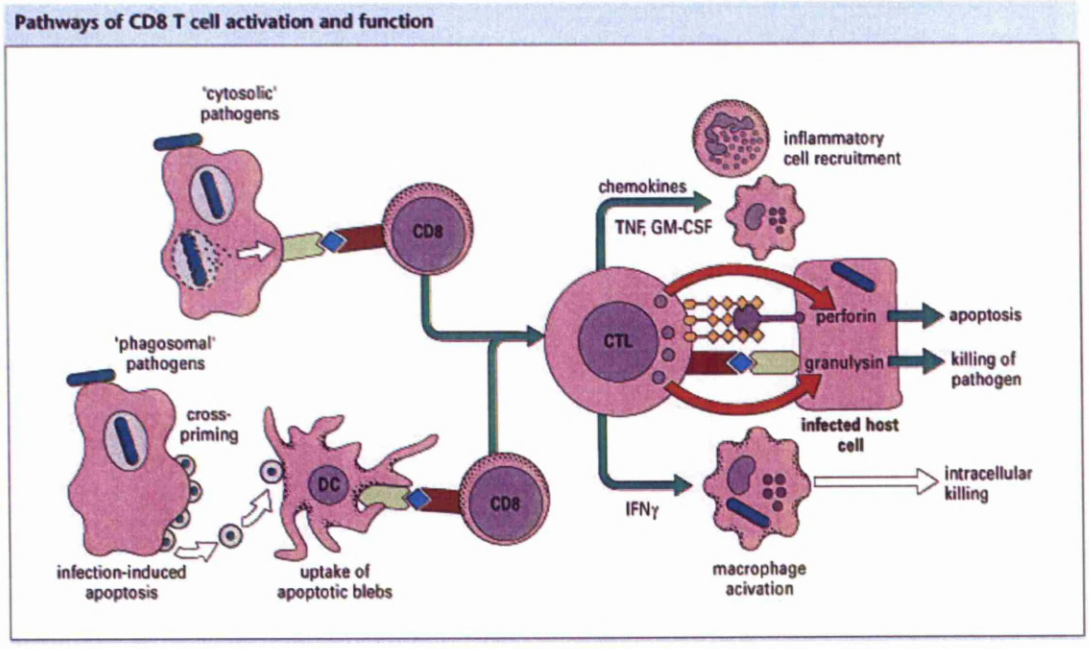


Figure 1.3: **CD8 T cell activation leads to release of chemokine TNF which causes inflammatory cell recruitment.** Adapted from Immunology[91], Male D, Brostoff J, Roth D, and Roitt I (editors), with permission from Elsevier Ltd, 2006.

There are immune consequences of male genital tract infections which include[92]:

1. Induction of an autoimmune response to spermatozoa;
2. Release of proinflammatory cytokines;
3. Activation of *yS* T lymphocytes;
4. Release of reactive oxygen species; and
5. Immune sensitisation to crossreacting antigens also expressed in spermheat shock protein.

1.5 Leucocytes in Male Infertility

Leucocytes are a normal constituent of semen irrespective of the fertility status of the male. The human ejaculate invariably contains cellular elements other than spermatozoa. These include epithelial cells from the reproductive tract and round cells; namely a mixture of immature germ cells and leucocytes. Advances in immunocytochemistry, using monoclonal antibodies, have made it possible to accurately differentiate leucocytes and their constituent subpopulations from other cells in semen. Such techniques make it possible to asses the role of leucocytes as an independent group of round cells in semen, including immune related infertility. Male germ cells develop and are sequestrated from the immune system in the testis by the blood testis barrier. Thus the male gamete resides for set periods of time in an immunologically privileged milieu. However, in the rete testes and epididymis no such barrier exists, and immunological and inflammatory responses are mounted against millions of spermatozoa stored in this organ.

Leucocytes in fertile ejaculate

Fertile men have leucocytes, in some cases with values considered as pathological, but without any effect on the seminal quality and fertility[93]. A previous study has shown that

every ejaculate from fertile and infertile men studied, contained granulocytes (the predominant leucocyte type), and monocytes / macrophages[94]. Around two thirds of all samples contained lymphocytes. The regular occurrence of leucocytes in the fertile ejaculates suggests that they play an important role in the normal function of semen. Certain components of semen may act as chemo-attractants for leucocytes. These may include products leaking from dead cells, complement components and bacterial products. In semen, leucocytes have an important phagocytic function, destroying necrotic spermatozoa and invading microorganisms. This ensures that the ejaculate contains predominantly healthy, viable spermatozoa. Distal portions of the male reproductive tract are not sterile, and the average ejaculate contains between five-six different species of bacteria[95]. To prevent bacterial colonisation at this site, leucocytes have an important role in immune surveillance at these sites, recognising and destroying invading microorganisms. Granulocytes and macrophages, bind to, endocytose and destroy factors with particular receptors including antibodies, complement factors and bacterial polysaccharides. No significant difference was found in the concentration of leucocytes or subpopulations of these cells (monocytes, lymphocytes and granulocytes) between fertile and sterile patients without ASA and immunological sterile groups (patients with genital tract infections excluded from the study)[48]. In a study by Gil and co-workers there was a predominance of macrophages / monocytes within the population of seminal leucocytes. No statistically significant difference was found in the absolute number of T and B lymphocytes between the three groups studied[48].

Leucocytes in infertile ejaculates

It is estimated that the frequency of leucocytospermia is between 10% and 20% among male infertility patients. A variation in leucocyte concentrations is a typical feature of human

semen, although it is considered pathological when it exceeds a concentration of >1x 10^6 WBC/ml semen, defined as leucocytospermia[13]. This concentration, however, represents only a minimal percentage of cells (0.4-4%) when compared to the normal number of spermatozoa, ranging from 25 to 250 million/ml[96]. In general, granulocytes represent 50% to 60% of all leucocytes in semen, macrophages 20% to 30% and T lymphocytes (CD4+ and CD8+) only 2% to 5%. Plasma cells and B lymphocytes are found rarely in semen[94]. However, another study found that macrophages were the predominant cell type in the infertile patients with or without leucocytopsermia[97]. In general, it is difficult to identify effects produced by a specific leucocyte type because there is always a mixture of different leucocyte populations in semen.

The incidence and significance of leucocytospermia amongst infertility patients is a much debated topic. Numerous studies in the past have found elevated concentrations of leucocytes to have a positive correlation with infertility. Using monoclonal antibodies, the first evidence was provided of a significant relationship between elevated leucocyte concentrations, their sub-types and a reduction in total number of spermatozoa, percentage motility, sperm velocity and total number of motile spermatozoa. When comparing infertility patients with and without leucocytospermia, men with many leucocytes in semen showed significant reductions in total sperm number, percent sperm motility, sperm velocity, motility index and total number of motile sperm. This was statistically significant in the high WBC / ejaculate group >3x10^6/ml [82].

Men with a high concentration of monocytes / macrophages were found to have a lower seminal volume, men with a high number of T cells were found to have a lower sperm velocity (although analysis of samples with elevated CD4+ T helper (>5x10^4/ml) or CD8+ T

cytotoxic lymphocytes (>$5x10^4$/ml) did not yield significant differences in any semen parameter, possibly because of low numbers of positive individuals), and men with a high granulocyte elastase concentration which is an indirect evidence for a high number of granulocytes, had a lower ejaculate volume, total sperm number and total motile sperm number[98]. The authors concluded that leucocytospermia may occur in male infertility patients and that high levels of leucocytes in semen are associated with poor semen quality.

A significant inverse relationship was found between the number of granulocytes in semen and a successful hamster ovum penetration test and increased leucocyte numbers were associated with reduced sperm concentrations[85]. Granulocytes in semen was found to inhibit sperm hyperactivation, an important step toward capacitation and acrosome reaction[99] and the number of leucocytes in semen was a strong predictor of IVF-ET failure[87].

The percentage of suboptimal semen specimens as defined by the IVF criteria (<10^7 total motile sperm per ejaculate) significantly increased with increasing seminal granulocyte concentrations. A twofold increased prevalence in such suboptimal semen specimens was observed at the leucocytopsermia threshold of $2x10^6$ granulocytes/ml[100]. However, other studies found no significant difference in leucocyte count between fertile and infertile ejaculates. Another study even argued that semen from fertile men contained significantly more leucocytes than subfertile men[101].

The exact cause of leucocytospermia may determine its effect on fertility. For example, declining frequency of ejaculation will lead to an increased proportion of ageing spermatozoa in the male reproductive tract. Spermatozoa will only be stored in the epididymis for up to a month, after which they are degraded by phagocytic leucocytes.

Increased numbers of granulocytes and macrophages infiltrate the reproductive tract for this function, and will be present in the following ejaculate. Such an ejaculate will be characterised as leucocytospermic, although the increased number of phagocytic leucocytes is actually the factor maintaining the quality of the ejaculate. Without this immune function, the ejaculate could contain an increased proportion of ageing spermatozoa, which are more likely to be functionally impaired, and / or carry genetic mutations.

Leucocytospermia is often described as the best indicator of genital tract inflammation, which itself is often clinically silent. It has been reported that 68% of leucocytopsermic patients with unexplained infertility were found to have clinically silent prostatitis. The level of the area affected is significant as it determines the amount of time leucocytes spend in contact with the sperm. Inflammation of the more proximal areas, including the epididymis, are likely to have increased significance, as the spermatozoa will be in prolonged and direct contact with leucocytes.

An indication of leucocytospermia as one of the causes of male infertility has come from studies on the role of oxidative stress in compromising the functional competence of human spermatozoa. Activated granulocytes can liberate large amounts of reactive oxygen species (ROS), proteases and cytokines which are important mediators of sperm damage in male genital tract inflammation[102]. Spermatazoa are particularly susceptible to such stress because of their high content of unsaturated fatty acids and they exhibit a highly specialised capacity for generating ROS including superoxide anion and hydrogen peroxide[103].

A number of retrospective analyses have indicated that the excessive generation of ROS is associated with defective sperm function particularly in oligozoospermia[104]. However, the

source of the high levels of ROS observed in cases of male infertility is unresolved and clinically significant. The washed human ejaculate contains, in addition to spermatozoa, a variable number of leucocytes particularly granulocytes which are powerful generators of ROS. Phagocytic leucocytes present in sperm suspensions can produce large amount of ROS[105]. If infiltrating leucocytes, rather than defective spermatozoa, are responsible for the oxidative stress observed in cases of male infertility, this will clearly have an impact on the therapeutic strategies that might be considered.

Since the influence of reactive oxygen species (ROS) on human spermatozoa was first reported in 1943, it is now widely accepted that oxidative stress is associated with male infertility. These highly reactive molecules cause lipid peroxidation of the polyunsaturated fatty acids present in sperm membranes which results in a dramatic loss of sperm oocyte fusion. At least 100 times more ROS are produced by leucocytes, particularly granulocytes than by spermatozoa[106].

ROS are considered to be responsible for much of the target cell and / or tissue damage induced by leucocytes. ROS induce lipid peroxidation within the cell membrane. When such lipid peroxides are exogenously added to sperm suspensions, an irreversible arrest of sperm motility occurs. ROS can also damage protein and nucleic acids[107]. There is good evidence that ROS generated by leucocytes can damage sperm motility[108], the sperm acrosome reaction[109], reduce fertilisation rates achieved by IVF[110] and correlates with reduced sperm function[111].

The great amount of energy necessary for the sperm journey is derived from mitochondria. Mitochondria possess their own genetic material which, unlike cellular DNA, is naked

(unprotected by histones and DNA binding proteins) and lack an efficient DNA repair mechanism. Thus the mitochondrial DNA is especially prone to damage elicited by the free radicals and ROS which are continuously generated by mitochondria during normal aerobic metabolism. A deletion in mitochondrial DNA would impair the synthesis of mitochondrial proteins necessary for mitochondrial function resulting in a subsequent decline in sperm motility[112].

The classical view of leucocytes was that only macrophages and polymorphonuclear neutrophils could initiate a respiratory burst to generate the superoxide needed for killing microorganisms. Evidence suggests that components of the oxidase system responsible for initiation of the burst and superoxide generation may also be present in both T and B lymphocytes[113]. There was no evidence that spermatozoa were the principal source of ROS production in any of the oligospermic samples examined[114].

Immunological causes of infertility have been associated with an increased production of ROS[115]. The purpose of the oxidase system in the B cell is unclear but it has been suggested that superoxide may play a role in the regulation of gene expression or digestion of the antigen. Semen samples that have ASAs present are likely to have increased numbers of B lymphocytes[114]. This means that increased ROS production is likely. Increased ROS production by semen samples in which ASAs are present may decrease fertility further.

Currently it is difficult to predict the impact of ASAs on fertility. Some men achieve fertilisation despite high semen antibody titres, whilst lower antibody titres may be the only abnormality detected in couples with prolonged infertility. Investigations into the extent of ROS induced damage, such as lipid peroxidation, may help to distinguish those patients who

are likely to experience complete failure of fertilisation. High levels of ROS production were associated with impaired sperm mucus penetration *in vitro*. As the level of ROS production increased, the number of progressively motile spermatozoa that were present in the mucus after both 15 min and 1 h was reduced[111]. It is unclear whether this was due to a direct effect of ROS on sperm function or if it was due to the fact that high ROS producing samples tended to have a lower sperm concentration.

Generally it appears that samples with increased ROS production tend to have poor semen quality together with reduced performance in a number of sperm function tests that are commonly used as a first step in male subfertility investigations. It is unclear if the poor performance can be accounted for by low sperm numbers or if this is exacerbated by oxidative damage that takes place in the epididymis or semen[111]. There was no relationship between the presence of antisperm antibodies and the production of ROS[111].

The results of the sperm recovery and motility maintenance test emphasise that spermatozoa are more vulnerable to ROS once they are denied the antioxidant defenses in seminal plasma and this needs to be taken into account during sperm preparation for assisted conception[116]. Leucocytes can be identified in sperm by using monoclonal antibodies through immunohistocytochemistry. The different leucocyte subsets can be identified using various cell surface markers or monoclonal antibodies. Table 1.4 outlines the cell surface markers used in this thesis.

Table 1.4: Human leucocytes can be identified by the following cell surface markers used in this thesis. Adapted with permission from Biochemical Society Transactions[117].

CD	Description	Leucocyte expression
CD3	Part of the T cell receptor complex	T cells
CD4	Ligand for class II MHC	Helper T cells (Th), macrophages
CD8	Ligand for class I MHC	Cytotoxic T cells (Tc)
CD14	Lipopolysaccharide-binding protein	Monocytes, macrophages
CD16	Receptor for complexed IgG	Neutrophils, NK cells, Monocytes, Macrophages
CD56	Neural cell adhesion molecule (N-CAM) isoform	NK cells, LGL
CD69	C- Type lectin protein	Activated T and B Lymphocytes

1.6 Cytokines in Male Infertility

Inflammatory disease has been long known to affect male reproductive function and is known to be associated with elevated levels of proinflammatory cytokines. Cytokines are polypeptides (regulatory proteins) usually smaller than 35 kDa, involved in haematopoiesis, immune cell development, inflammation and immune responses. Several cytokines have direct effects on testicular cell functions, and a number are produced within the testis even in the absence of inflammation or immune activation events. There is compelling evidence that cytokines, in fact, play an important regulatory role in the development and normal function of the testis. There are two major roles that cytokines play in testis: the first is as mediators of pathophysiological outcomes of immune–endocrine interactions during inflammatory disease; the second is as growth and differentiation factors that help orchestrate cellular interactions during normal physiological functions.

1.6.1 An overview of cytokines discussed in this thesis

Table 1.5 gives an overview of major cytokine families including the source, their principal targets and the principal effects.

The cytokine milieu is critical for determining the outcome of an immune response following B cell T cell interaction. Th1 cells are $CD4^+$ that produce IL-2 and IFN-g and are chiefly responsible for delayed type hypersensitivity responses. Th2 cells are $CD4^+$ that produce IL-4, IL-5, IL-10 and IL-13 and are very efficient helper cells for the production of antibodies, especially IgG and IgE. Figure 1.4 outlines the different cytokines which are produced by Th1 and Th2 cells. These cytokines also play a role in B cell development.

Table 1.5: Description of the major cytokines. Adapted from the Cytokine Handbook. Thomson AW & Lotze MT (editors), permission from Elseiver Ltd 2003.

The Major Cytokines				
Cytokine	**Immune system source**	**Other cells**	**Principal targets**	**Principal effects**
IL-6	T cells, B cells	fibroblasts, macrophages	B cells, hepatocytes	B cell differentiation, induces acute phase proteins
IL-8	monocytes	fibroblasts	neutrophils, basophils, T cells, keratinocytes	chemotaxis, angiogenesis, superoxide release, granule release
IL-10	T cells		TH1 cells	inhibition of cytokine synthesis
IL-11		bone marrow stromal cells, fibroblasts	hemopoietic progenitors, osteoclasts	osteoclast formation, colony stimulating factor, elevates platelet count in vivo, inhibits proinflammatory cytokine production
IL-12		monocytes	T cells	induction of Th1 cells
TNF-α	macrophages, mast cells, lymphocytes		macrophages, granulocytes, tissue cells	activation of macrophages, granulocytes and cytotoxic cells, leukocyte/endothelial cell adhesion, cachexia, pyrexia, induction of acute phase protein, stimulation of angiogenesis, enhanced MHC class I production
IFN-γ	T cells, NK cells	epithelia, fibroblasts	leukocytes, tissue cells, Th2 cells	MHC class I and II induction, macrophage activation, ↑ endothelial cell/lymphocyte adhesion, MØ cytokine synthesis, antiviral state, antiproliferative (Th1 cells)

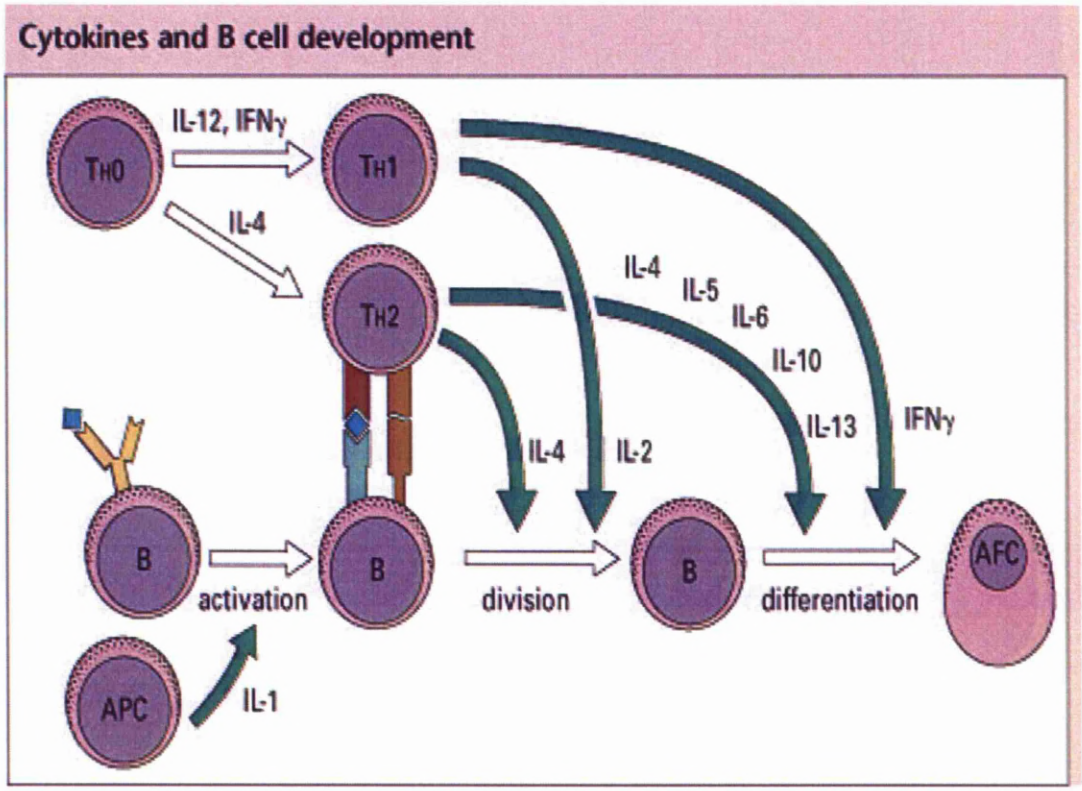


Figure 1.4: Cytokine secretion from CD4$^+$ T cells controls B cell proliferation and differentiation. AFC- antibody-forming cell (B cell), APC-antigen presenting cell[91]. Adapted from Immunology. Male D, Brostoff J, Roth D, and Roitt I (editors), with permission from Elsevier Ltd, 2006.

Spermatogenesis is a complex process regulated by endocrine and testicular paracrine / autocrine factors. Gonadotrophin releasing hormone (GnRH), which is released from the hypothalamus, affects the release of leutinizing hormone (LH) and follicular stimulating hormone (FSH) from the hypophysis. LH induces Leydig cells to produce testosterone, which under high levels negatively affect LH production (through the hypothalamus or hypophysis). On the other hand, FSH affects Sertoli cells in the seminiferous tubules to produce various autocrine / paracrine factors such as transferrin, androgen-binding protein, cytokines and inhibin. Under high levels, these factors (mainly inhibin) negatively affect FSH production through the hypophysis. In addition, LH and FSH could affect, directly or indirectly, the production of various paracrine / autocrine factors from testicular cells of the interstitial and seminiferous tubule cells (Figure 1.5).

Gonadotropins are involved in the regulation of several testicular paracrine factors, mainly of the IL-1 family and testicular hormones. Testicular cytokines and growth factors (such as IL-1, IL-6, TNF, IFN-γ, LIF and SCF) have been shown to affect both the germ cell proliferation and the Leydig and Sertoli cells functions and secretion[118]. These cytokines also control the secretion of the gonadotropins and testosterone in the testis. Under pathological conditions the levels of proinflammatory cytokines are increased and negatively affect spermatogenesis. Autocrine and / or paracrine factors affect testicular functions and also regulate the secreted levels of endocrine factors. Under pathological conditions, such as systemic or testicular infection / inflammation, the levels of proinflammatory cytokines in the testicular tissue could be increased and thus disrupt the balance and normal levels of these cytokines in the testis and also the levels of the secreted endocrine factors (Figure 1.6).

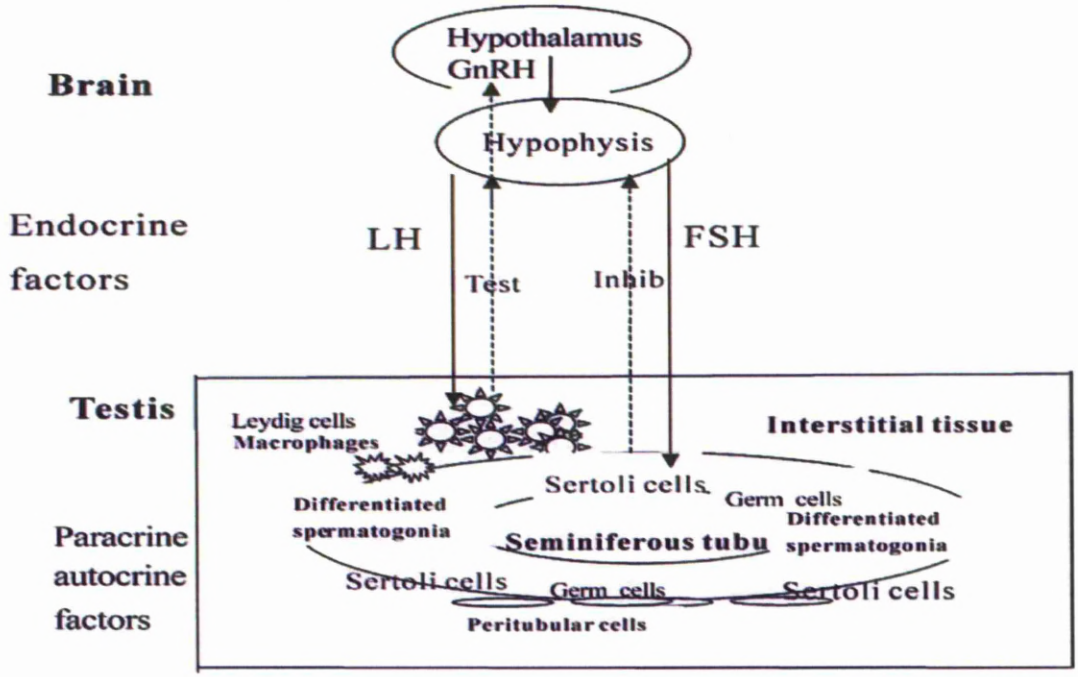


Figure 1.5: Endocrine control of spermatogenesis. Reprinted with permission from Asian Journal of Andrology[118].

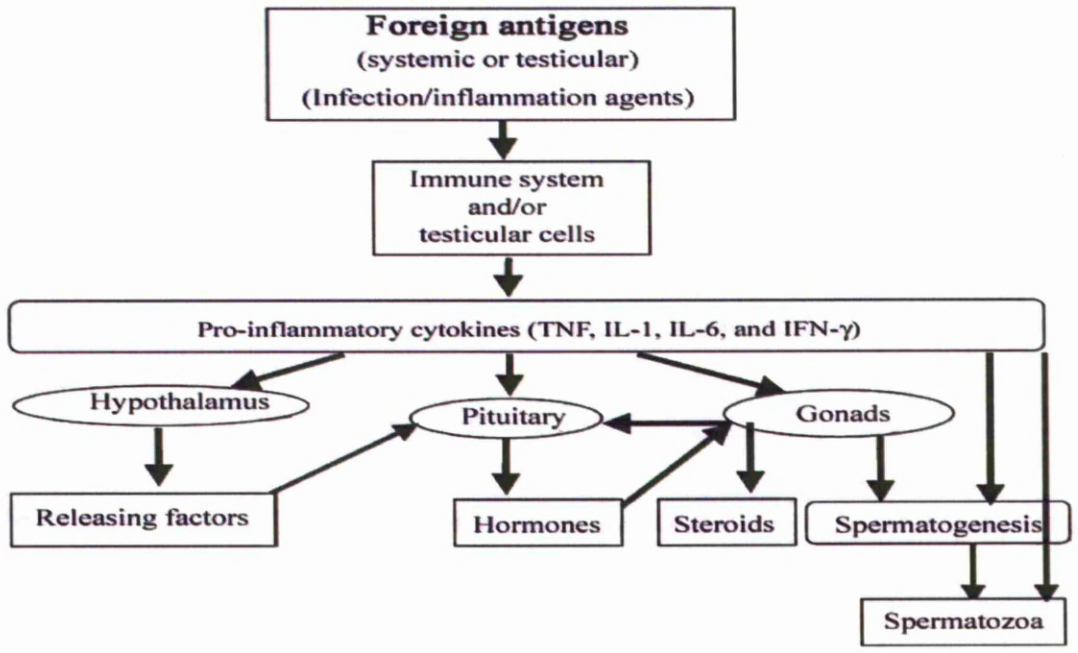


Figure 1.6: Cytokine influence on spermatogenesis. Reprinted with permission from Asian Journal of Andrology[118].

Cytokines also affect testicular function via the hypothalamic-pituitary-adrenal pathway. Neuroimmune endocrine interactions are evident during stress related events and have been demonstrated in experimental models that utilise local application of cytokines to the brain. Another neural mechanism directing function of testicular cells involves autonomic nerves in the testis that appear to influence steroidogenesis and spermatogeneis directly or via vascular function. Inhibitory effects of the neuroimmune-endocrine pathway occur over a longer period of time course, while the autonomic nerves in the testis appear to be responsible for immediate alterations in testicular function[119].

The male reproductive tract is an immunologically dynamic tissue capable of initiating both humoral and cell-mediated immune responses. Sperm cells express unique differentiation antigens capable of eliciting autoimmune responses. These cellular immune responses are mediated by cytokines. There is considerable evidence that cytokines play a role in immunological infertility. APC are a specialised group of cells termed antigen presenting cells which link the innate and adaptive immune systems by producing cytokines. These cells carry antigens in a form that can stimulate lymphocytes (Figure 1.7).

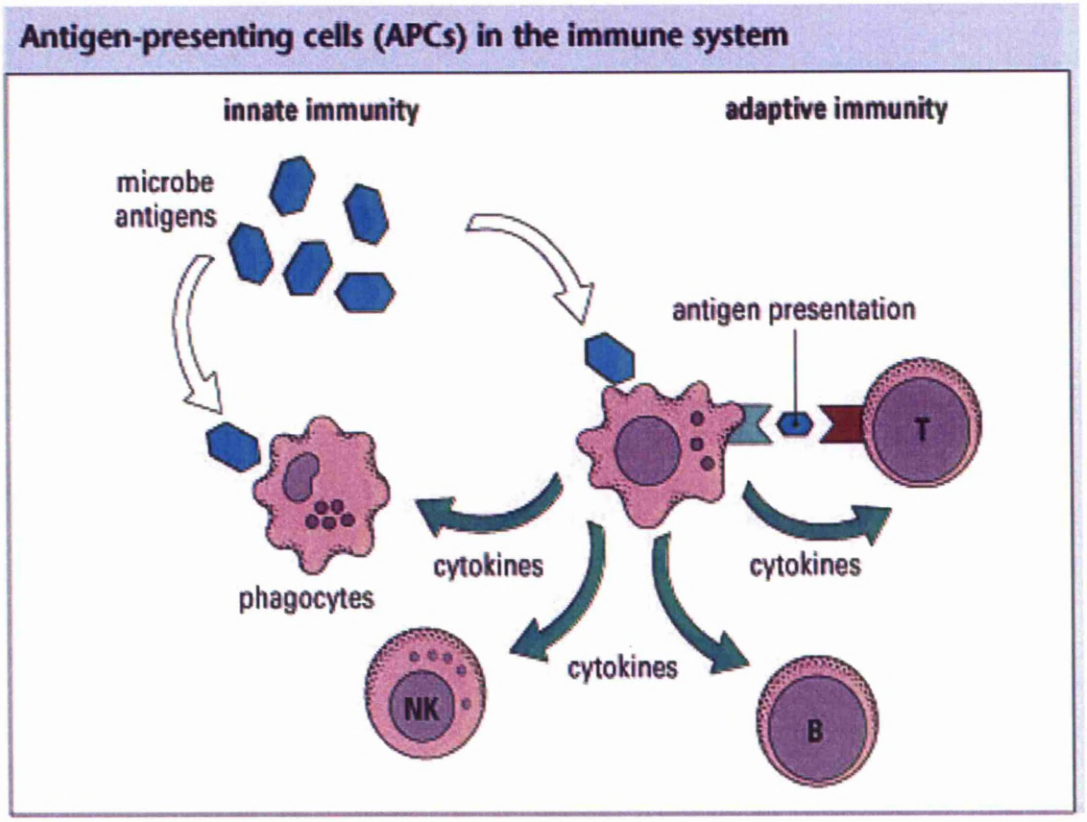


Figure 1.7: APCs link the innate and adaptive immune systems[91]. Adapted from Immunology. Male D, Brostoff J, Roth D, and Roitt I (editors), with permission from Elsevier Ltd, 2006.

1.6.1.1 Interleukin-6 (IL-6)

Interleukin-6 (IL-6), a 30 kDa glycoprotein, is part of a family of cytokines that act through the gp130 receptor, and is an extremely important cytokine in the regulation of inflammation and immunity[120] that is present at low levels in the circulation of healthy adults[121] .

Synthesis and regulation: Many cells including T and B cells, macrophages, fibroblasts and endothelial cells produce IL-6. In the testis, IL-6 is produced by Sertoli cells in response to stimulation by FSH, testosterone, neuropeptides, and residual bodies[122;123]. Leydig cells also express IL-6 after LH stimulation in vitro[124;125].

Receptors and signalling: IL-6 binds to a plasma membrane receptor, IL-6 receptor. The IL-6 receptor is expressed on activated B cells, plasma cells, T cells, monocytes, epithelial cells, fibroblasts, hepatocytes and neural cells. Soluble forms of IL-6Ra and gp130 are found in human serum may play a role in modulating the response to IL-6[126].

Actions:

T and B cells: IL-6 acts synergistically with IL-1 and TNF-α to induce T cell proliferation and differentiation. It also stimulates B cells to differentiate into plasma cells. This proinflammatory role may be important in chronic infections in order to lead to the development of specific humoral and cellular immune responses[127].

Testis: IL-6 has a number of effects on seminiferous epithelial function, including stimulation of transferrin production by Sertoli cells[128], and inhibition of meiotic DNA synthesis in pre-leptotene spermatocytes[129]. Both LPS and IL-1 stimulate IL-6 production by Sertoli and Leydig cells indicating that both cytokines are increased within the testis under inflammatory conditions[123-125].

1.6.1.2 Interleukin-8 (IL-8)

Synthesis and regulation: IL-8, a 77 amino acid glycoprotein dimer, is synthesised as a 99 amino acid precursor by activated monocytes, lymphocytes, fibroblasts, epithelial cells and hepatocytes. It can be induced by IL-1 β, TNF-α, LPS and viral RNA. LPS is particularly effective in inducing production of IL-8 by monocytes. IFN-γ inhibits the production of IL-8.

Receptors and signalling: IL-8 acts through two high affinity G-protein coupled receptors, IL-8 receptor type A and IL-8 receptor type-B[130].

Actions: IL-8 is important for cell-mediated response to infection. It is a chemotactic cytokine that activates neutrophils, leading to degranulation and release of collagenase and elastase containing granules[131].

1.6.1.3 Interleukin-10 (IL-10)

IL-10 is an important deactivating, immunosuppressive and anti-inflammatory cytokine.

Synthesis and regulation: IL-10, a 18.5kDa homodimer of two polypeptide chains is produced by macrophages, T and B cells and various other cells such as mast cells and keratinocytes[132]. LPS, catecholamines and glucocorticoids are known to increase IL-10 secretion[133].

Receptors and signalling: The IL-10 receptor has two subunits which are members of the cytokine receptor family type 2[134] and signals via a JAK kinase pathway.

Actions:

Monocytes and macrophages: IL-10 inhibits the production of proinflammatory cytokines such as IL-1 β, IL-6, IL-8, and TNF-α by activated monocytes and macrophages[135]. IL-10 inhibits the production of prostaglandin E2 (PGE2) through downregulation of cyclooxygenase-2 (COX-2) expression. This in turn reduces the expression of the enzymes involved in cervical changes (matrix metalloproteases), which are regulated by a PGE2-cAMP pathway. IL-10 has been shown to down regulate MHC class II molecules at the

monocyte cell surface through post translational effects[136]. IL-10 inhibits the transport of MHC class II molecules to the cell surface.

Neutrophils: IL-10 inhibits the LPS induced production of IL-1 β, IL-8 and TNF-α by neutrophils. IL-10 blocks the gene transcription of these proinflammatory cytokines by inhibiting NF*k*B activation. It also suppresses the killing of phagocytosed bacteria and enhances the production of IL-RA[137].

T and B cells: IL-10 enhances expression of MHC class II molecules on B cells. It also increases antibody production and B cell survival. IL-10 strongly inhibits CD4+ cytokine production, whilst stimulating CD8+ cells into cytotoxic activity and proliferation.

1.6.1.4 Interleukin-11 (IL-11)

IL-11 is a multifunctional cytokine first isolated in 1990 from bone marrow derived stromal cells. It is a key regulator of multiple events in hematopoiesis, most notably the stimulation of megakaryocyte maturation. It is also known under the names adipogenesis inhibitory factor (AGIF) and oprelvekin.

In reproduction:

IL-11 and leukaemia inhibitory factor (LIF) are produced by the endometrium and are absolutely required for implantation in mice. Both cytokines increase adhesion of primary human endometrial epithelial cells to fibronectin and collagen IV. A study demonstrated that targeting IL-11 and LIF may be useful in regulating fertility by either enhancing or blocking implantation[138]. Defects in IL-11 signalling in mice result in trophoblast over-invasion and fetal loss[139].

IL-11 has been shown to be significantly higher in oligo-terato-asthenozoospermic infertile patients. A study also found that a combined determination of IL-6, IL-8 and IL-11 in the

seminal plasma of men with genital infection and oligo- terato- asthenozoospermia may provide clinically useful information for the diagnosis of male accessory gland infection[140].

1.6.1.5 Interleukin-12 (IL-12)

IL-12, a heterodimeric protein, enhances Th1 immunity and is important for defence against intracellular pathogens.

Synthesis and regulation: IL-12 is produced by B cells and dendritic cells, in response to stimulation by T cells and antigens or bacteria. It is also produced in response to LPS stimulated macrophages, microglia, neutrophils and astrocytes.

Receptors and signalling: The IL-12 receptor is a high affinity receptor with 2 chains, β 1 and β 2. IL-10 inhibits production of IL-12 by monocytes

Actions: IL-12 acts in synergy with IL-2 to induce IFN production by T cells and NK cells[141]. It enhances NK cell activity and costimulates blood lymphocyte proliferation. It also stimulates the proliferation and activation of Th1 cells[142].

1.6.1.6 Tumour Necrosis factor alpha (TNF-α)

TNF-α is a proinflammatory, cytotoxic cytokine with a trimeric structure, which acts via receptors that mediate a cell-death signal (Figure 1.8).

Synthesis and regulation: TNF-α can be produced by many cells including monocytes, T and B cells, neutrophils, glia, neurones and smooth muscle cells, but is mainly secreted by activated macrophages[143]. Production of TNF-α can be stimulated by a variety of chemical, biological and physical factors. Viruses and bacterial products such as endotoxin, cytokines such as IL-1, IFN-γ, GM-CSF, X-ray radiation, trauma and ischaemia can all induce TNF-α production. The synthesis of TNF-α is tightly controlled. In the normal healthy human, it is produced in very small quantities so that there is virtually no TNF-α detectable. However, it

is one of the first cytokines to be produced in response to stimulation such as sepsis or trauma[144].

TNF-α has been found in pachytene spermatocytes and round spermatids[145], and in activated macrophages isolated from the testis[146]. TNF-α exists in two forms. Most biological activity of TNF-α is attributed to the soluble form but the membrane bound form TNF-α does have some activity and is more able to activate TNF receptor 2[147]. TNF-α production is regulated during transcription (NF*k*B promotes[148] and corticosteroids and IL-10 inhibits), translation and at a post-translational stage, TNF-α is secreted as a prohormone and is proteolytically cleaved by TNF-α coverting enzyme (TCE).

Receptors and signalling: There are two TNF high affinity receptors, found on most cell types apart from red blood cells. TNFR1 is thought to be responsible for most TNF-α biological activity. TNFR2 is thought to potentiate the effects of TNFR1, promote the proliferation of T cells and in oligodendrocyte regeneration[143;149;150].

Actions:

Systemic: TNF-α produces fever and anorexia due to its effects on the hypothalamus. It also leads to release of acute phase proteins from the liver. TNF-α is a procoagulant and increases permeability and MHC class I expression of vascular endothelium which can lead to disseminated intravascular coagulation and shock.

Monocytes and macrophages: TNF-α acts synergistically with IFN-γ in inducing production of IL-1, IL-6, IL-8, GM-CSF, IFN-γ and TGF-β. It also induces further release of TNF-α. TNF-α causes degranulation, superoxide release and increased adhesion in granulocytes.

T and B cells: In B cells, TNF-α induces superoxide production. In T cells it induces cytotoxic invasiveness.

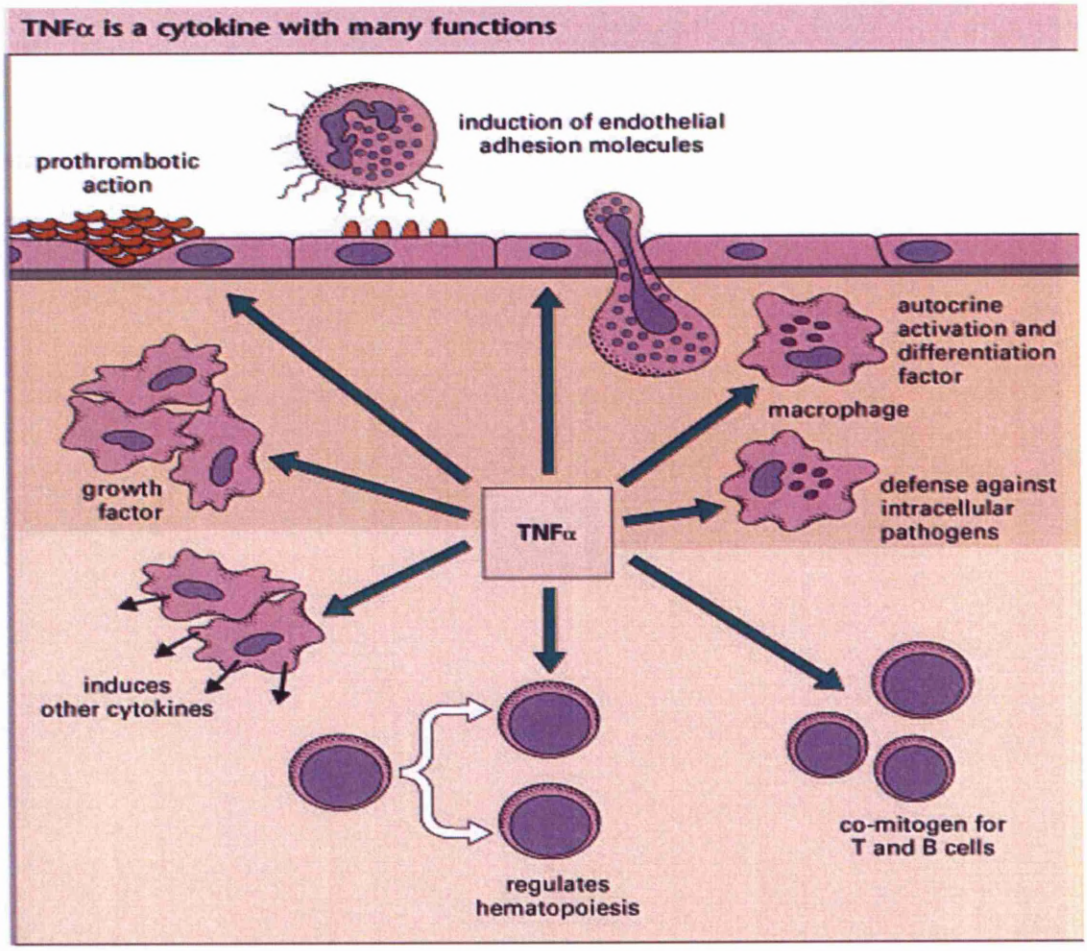


Figure 1.8: TNF-α; has several functions in inflammation. It is prothrombotic and promotes leukocyte adhesion and migration (top). It has an important role in the regulation of macrophage activation and immune responses in tissues (centre), and it also modulates hematopoiesis and lymphocyte development (bottom)[91]. Adapted from Immunology. Male D, Brostoff J, Roth D, and Roitt I (editors), with permission from Elsevier Ltd, 2006.

Testis: Similar to IL-1, TNF-α inhibits Leydig cell steroidogenesis[151], and its localisation to the post-meiotic germ cells also indicates possible involvement in the process of spermatogenesis. For example, TNF-α might play a role in controlling the efficiency of the spermatogenic process, inhibiting germ cell apoptosis by regulating the level of type 2 transmembrane protein Fas ligand (FasL)[152]. In pathology, TNF-α has been implicated as a major causative agent in the development of autoimmune orchitis[153].

1.6.1.7 Interferons

Interferons (IFN) are a very large family of cytokines with potent anti-viral actions, which are classified as type I (IFN-α or IFN-β) or type II (IFNγ) (Figure 1.9). IFN-γ is normally produced by T lymphocytes and plays an immunoregulatory role by stimulating antigen specific immune responses[154]. Both type I and type II IFNs have been found in the testis, and are stimulated by viral infections, particularly in Sertoli and Leydig cells[155;156].

Interferons have been shown to inhibit Leydig cell steroidogenesis *in vitro*, through inhibition of StAR expression[157]. IFN-γ exerts its inhibitory effects on testosterone production at the level of cholesterol transport into the mitochondria and expression of both P450scc (cholesterol side chain cleavage enzyme) and P450c17 (cytochrome enzyme involved in steroid synthesis)[158]. Moreover, IFN-γ has been implicated in the onset of many autoimmune diseases, including autoimmune orchitis[159]. Furthermore, there exists a complex interaction of cytokines produced by Th1 and Th2 cells in the regulation of macrophage activation and stimulation of B lymphocytes. Figure 1.10 highlights these interactions.

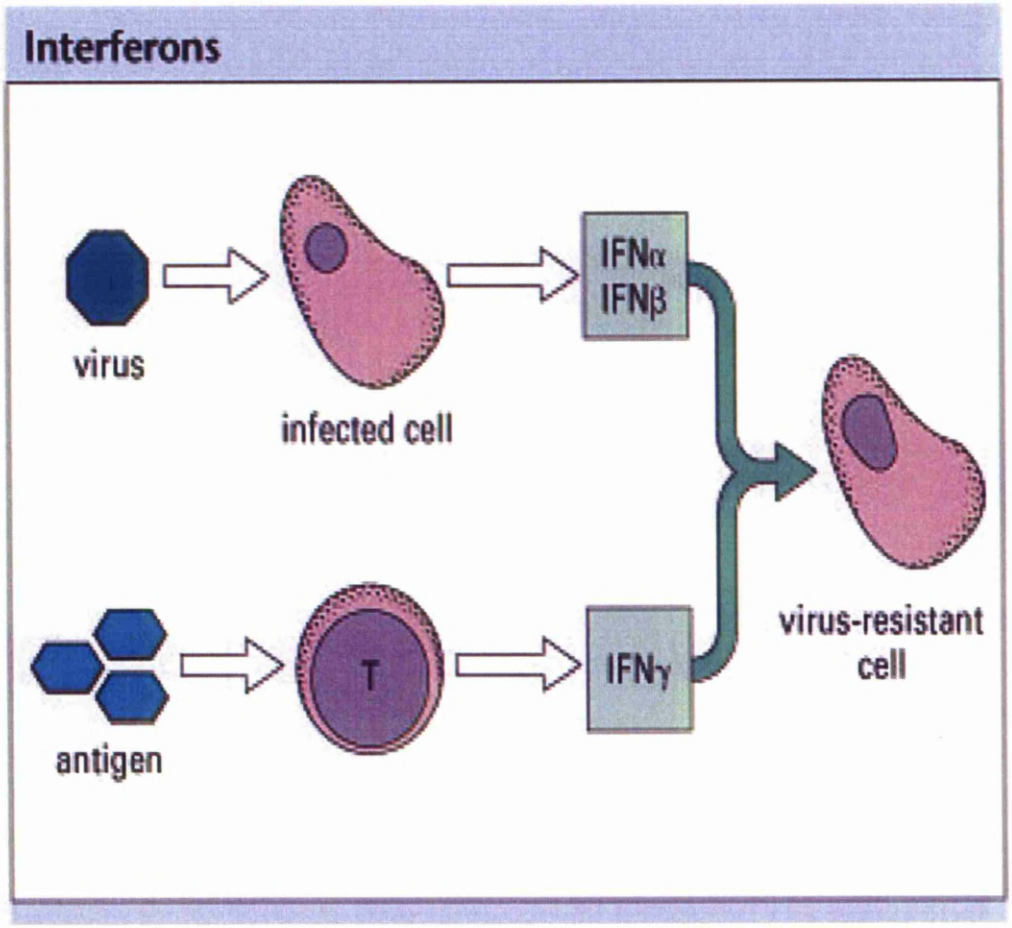


Figure 1.9: Interferon-α (IFN-α) and/or interferon-β (IFN-β) secreted by the virus infected cells and interferon-γ (IFN-γ) secreted by the TH1 cells are involved in protecting the testis by inducing resistance against viral infections[91]. Adapted from Immunology. Male D, Brostoff J, Roth D, and Roitt I (editors), with permission from Elsevier Ltd, 2006.

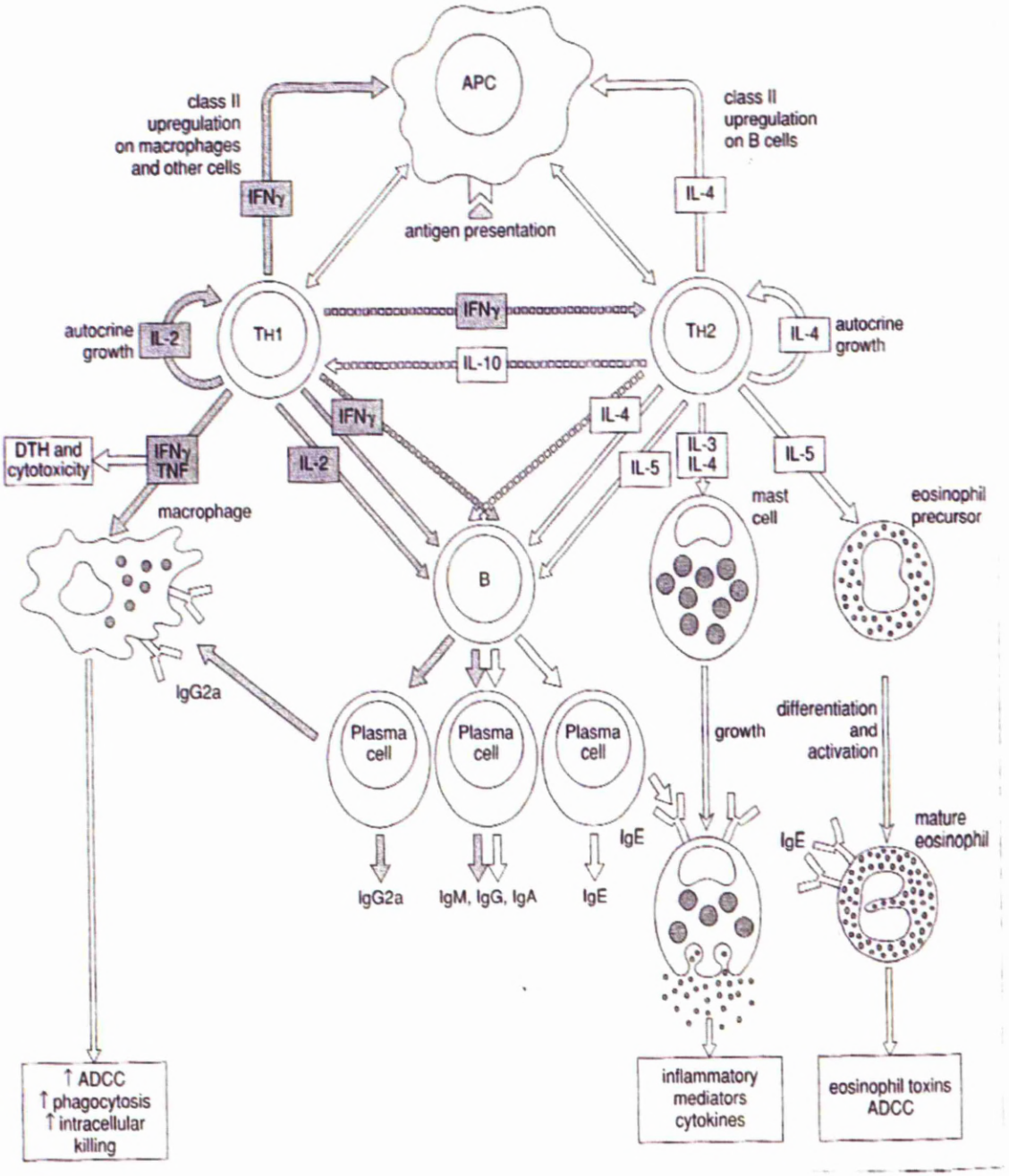


Figure 1.10: Major effect of cytokines produced by Th1 and Th2 cells[160]. Reprinted with permission from Immunobiology. Janeway CA, Travers P, Walport M, Shlomchik M (editors), Churchill Livingstone.

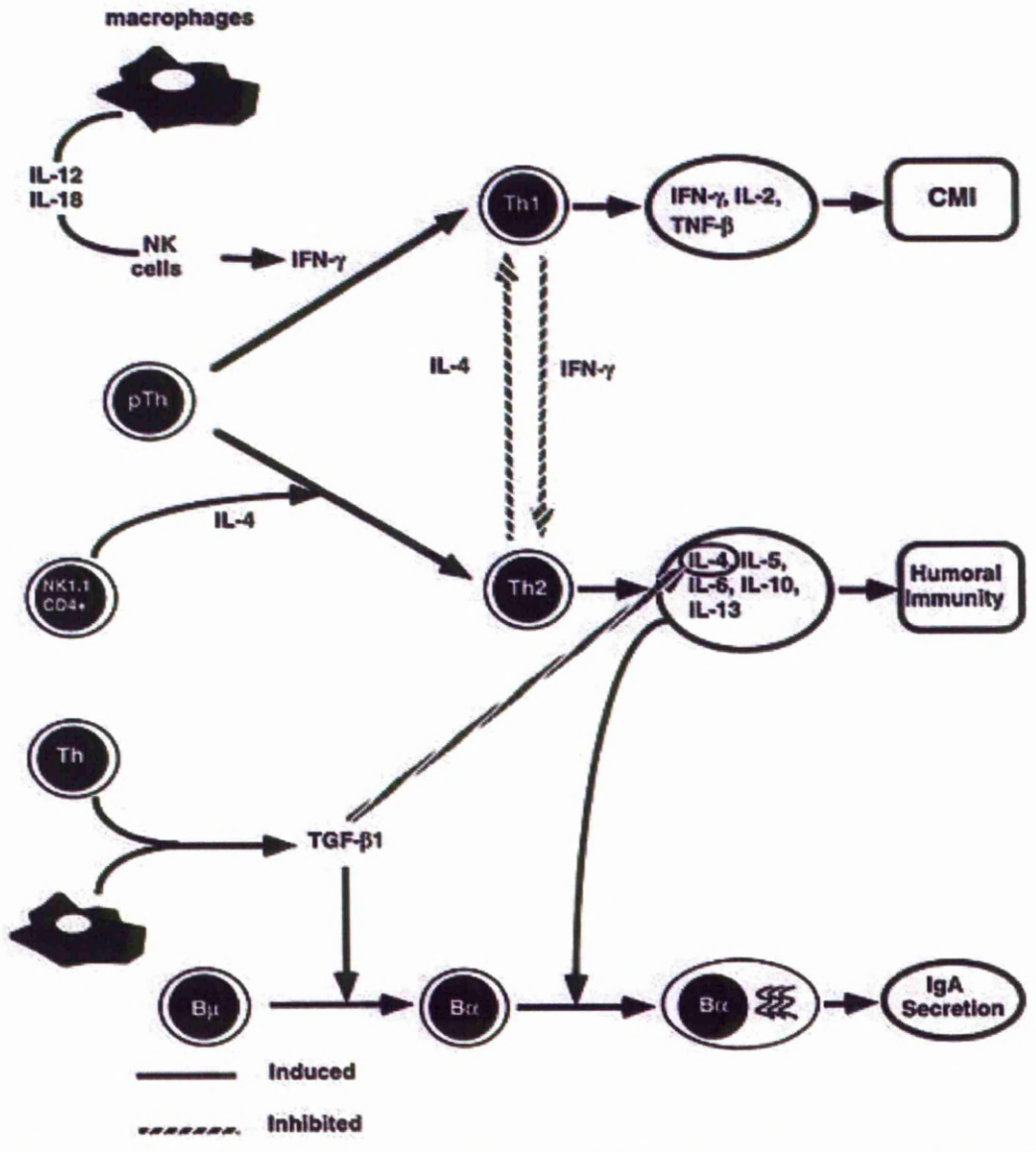


Figure 1.11: The role of cytokines in Humoral & Cell mediated Immunity[161;162]. Reprinted with permission from Mucosal Immunology. Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR (eds). San Diego: Academic Press.

Cytokines also have a role to play in humoral immunity and Figure 1.11 shows the interaction of cytokines in cell mediated immunity and humoral immunity. Cytokines are detrimental to murine embryonic development *in vitro* and IFN and TNF have inhibitory effects on human sperm motility as well as performance in sperm penetration assays[163]. These cytokines, if present, may adversely affect sperm cell function and the reproductive process. Additional efforts are needed to clarify the effects of cytokines on fertility.

1.7 Immune disorders & Reproductive failure

Taking into consideration the various immune system disorders, they may lead to reproductive failure at different stages of the reproductive process: unexplained infertility, recurrent IVF or ICSI failures and recurrent miscarriages. Two immune cells have been associated with infertility, recurrent implantation failures and recurrent miscarriages. These are $CD56^+$ natural killer cells[164;165] which produce cytokines including TNF-α and $CD19^+$ (B cells) which produce antibodies to hormones such as HCG, progesterone etc.

Cytokines are released by various immunocompetent cells in the male urogenital tract. They play an important role in cell signalling and perform broad pleomorphic activities[166]. Studies have reported that cytokines may be mediators of oxidative stress and have the potential to alter the redox (reduction-oxidation reaction) equilibrium[167-169]. A study in patients with genital tract inflammation reported that cytokines may modulate pro-oxidant and anti-oxidant activities in the male genital tract[170]. Cytokines are also capable of influencing sperm function and fertility[77;171]. Chronic urogenital infections or humoral immune responses have been suggested to play a role in reduced sperm quality and fertility[154]. Tissue damage caused by microbial pathogens is also associated with the secretion of proinflammatory cytokines[172]. Regulatory factors released in the reproductive tracts are able to influence sperm function

directly or indirectly. Cytokines produced by activated neutrophils were found to adversely affect sperm motility and sperm fertilising ability in the hamster ovum penetration test[39]. The same group also reported that IFN-γ and TNF-α significantly reduced sperm motility *in vitro*.

Controversy exists over the role of cytokines in reproductive failure as studies have shown that TNF-α *in vitro* does not affect human sperm motility, hamster ova penetration, mouse *in vitro* fertilisation and embryo development[173], high concentrations of TNF-α and IL-2 are not related to sperm parameters or to the presence of bacterial agents[174] and IFN-γ, TNF-α or IL-8 has no effect on the calcium ionophore challenged acrosome reaction *in vitro*[175]. The total number of motile spermatozoa is considered a clinically important parameter with regards to fertilisation rates in IVF programmes[176]. It has been suggested that IL-1, IL-6 and TNF-α might influence sperm motility via direct or indirect effects such as reduced mucosal penetration properties and therefore reduced male fertility[177]. These cytokines may result in decreased sperm motility and therefore, in reduced ova-penetrating properties. Positive correlations have been demonstrated between sperm motility and sperm morphology and fertilisation rates in IVF which are significant factors in predicting both fertilisation and pregnancy rates[110;178]. However, some studies have shown that IL-6 provides a positive signal in enhancing the fertilising capacity of human sperm by increasing capacitation leading to the acrosome reaction[179]. Recently, seminal plasma has been found to regulate endometrial cytokine expression, leucocyte recruitment and embryo development in the pig[180].

Considerable research over the past 10 years has helped to identify a role for several cytokines in the regulation of the male reproductive tract, particularly within the testis. This apparent overlap between testicular and immune regulatory mechanisms could provide the

key to understanding both the processes leading to inflammation-mediated damage of testicular function and the phenomenon of immune privilege in the testis.

Regardless of the importance of their role in normal testicular physiology, it is evident that several cytokines are shared by the immunological and testicular systems. As a result, activation or failure of the immune system can result in perturbation of testicular function at least partially through cytokine-mediated effects. For example, up-regulation of IL-1 and IL-6 by inflammation in either the circulation or within the testis itself would undoubtedly disrupt the ability of these cytokines to play a role in the fine control in the regulation of the spermatogenic cycle. Similarly, the production of IFNs or TNF-α during a viral or bacterial infection could suppress steroidogenesis, due to the specific effects of these cytokines on Leydig cells. Consequently, excessive production of cytokines within the testis may be an important cause of the impairment of spermatogenesis and steroidogeneis that usually occurs during inflammation, illness and injury. Figure 1.12 highlights the complexity of the cytokine regulatory network within the testis, and the overlap with cytokines produced by immune cells as wellas somatic cells during immune and inflammatory responses.

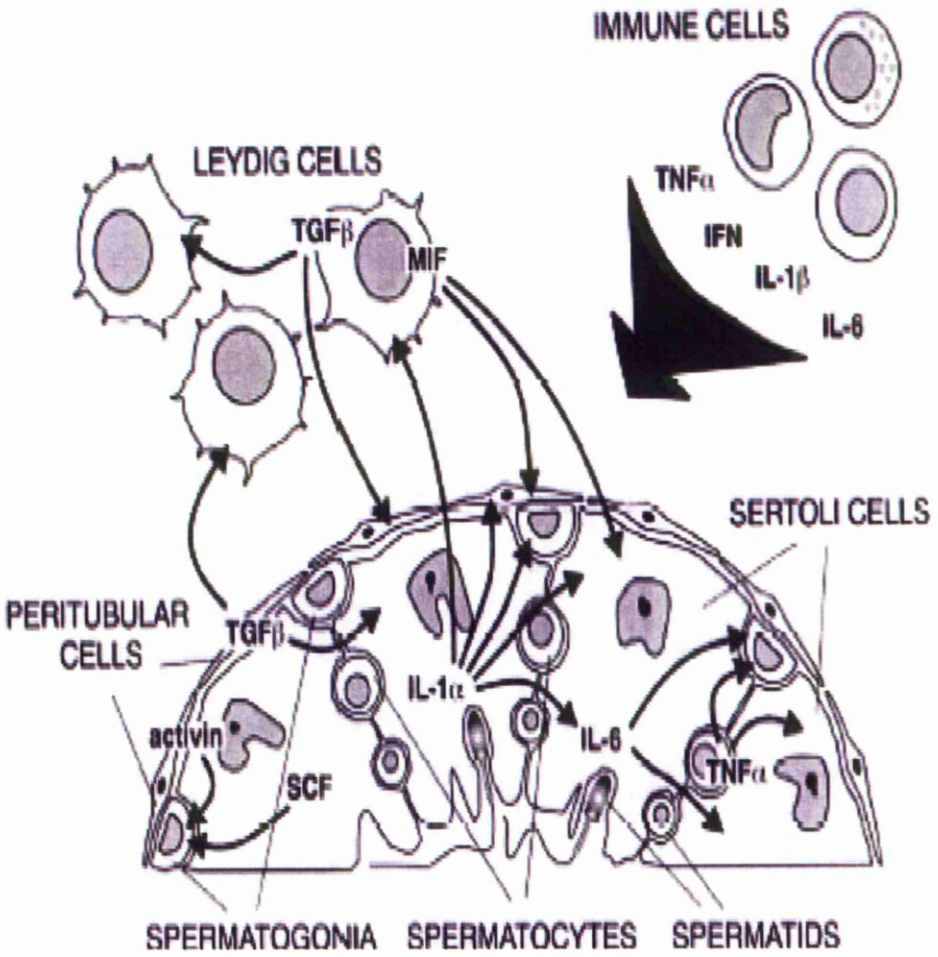


Figure 1.12: Diagrammatic representation of cellular relationships and sites of production and action of key cytokines within the mammalian testis[181]. Reprinted with permission from Journal of Reproductive Immunology.

1.8 Summary

Application of newly developed molecular techniques to the field of male infertility has made the delineation of subtle causes of infertility—an unapparent genital tract infection, immune system activation within the genital tract, mutations in sperm mitochondrial or chromosomal DNA, alterations in sperm components involved in receptor-ligand interactions, and production of sperm autoantibodies—all increasingly amenable to clinical analysis. Continued investigations at the cellular and molecular level of the causes of male infertility will also lead to novel treatment regimens as well as development of new methods of fertility regulation.

Investigations on the potential role of ASAs in infertile couples have been performed extensively during the past three decades. However, questions about the underlying mechanisms of ASA in reproduction, testing methods, the relationship between ASAs and infertility, and the treatment of ASA-mediated human infertility still remain. In fact, research on ASA-related infertility is limited. In conclusion, sperm antigens and ASAs appear to have both adverse and favorable effects, with some even having no effect on fertility. But when do they act on fertility, and to what extent do they affect fertility? This study aims to answer some of these questions.

Leucocytospermia is said to occur in 10–20% of infertile patients. It has been considered to be a significant cofactor in poor semen parameters. The relationship between leucocyte concentrations in semen and abnormal semen parameters has not been clearly established. The clinical significance of leucocytospermia has been questionable particularly if the patients are asymptomatic. Some authors have even concluded that the threshold of clinically significant leucocyte count in semen should be estimated at a higher level than the current WHO recommendations[100]. If leucocytospermia was considered to be secondary to infection

then one would have anticipated that antibiotic treatment would reduce the leucocyte count and improve sperm parameters. However, there was no beneficial effect of antibiotic treatment on semen parameters nor were there a statistically significant reduction in the leucocyte count amongst both the leucocytospermic and the non leucocytospermic individuals[182]. However, other authors have suggested that semen parameters including progressive motility rate and sperm concentrations were significantly lower in the leucocytospermic group compared with the control group. Other semen parameters were not affected by the presence of leucocytes[183].

ICSI outcome, including fertilisation (82% vs. 87%) and embryo development rates (79% vs. 86%) were significantly lower in the leucocytospermic group compared with the control group although there were no statistical differences for embryo quality, embryo cleavage and pregnancy rates[183]. These results indicate that some semen parameters and the outcome of ICSI were negatively affected by the presence of leucocytospermia. In contrast to the above study, another study reported fertilisation rates of 63.4% (range 44.4–87.5%) in nonleukocytospermic couples and 64.3% (range 45.3–100.0%) in leukocytospermic couples, and the corresponding pregnancy rates were 34.5% and 50%, respectively. These results show that leukocytospermia may not necessarily have a negative effect on outcome after either *in vitro* fertilisation or intracytoplasmic sperm injection[184].

Even though biologically active cytokines are found in abundance in seminal plasma and have been shown to be secreted by spermatozoa, the function of the cytokine network in relation to the fertilising capacity of the sperm has never been evaluated. Furthermore, the role of the cytokine network in male subfertility is also poorly understood. In most studies, only a few cytokines (up to four) in seminal plasma have been measured, and the

pathophysiological effect of these cytokines on fertilisation rates and pregnancy outcomes remain largely unexplored.

1.9 Project Aims

Given the controversy that exists about the role of leucocytes and cytokines in male subfertility, the main hypothesis of this thesis was that neither leucocytospermia on its own nor individual cytokines have any significant role to play in male subfertility. However, the combination of leucocytospermia in the presence of the cytokine network may adversely affect sperm parameters and lead to male subfertility. In this study, leucocytes and seven cytokines have been analysed at the same time in the seminal plasma of both subfertile men and in men attending for IVF. This was used to determine if either leucocytospermia or individual cytokines had an influence on sperm parameters / fertilisation rates and pregnancy outcomes in an IVF cycle.

Chapter 2, the materials and methods chapter, discusses the various methodological aspects of the study ranging from ethical approval, patient recruitment, immunohistocytochemistry and ELISA techniques and statistical analysis.

Chapter 3, the leucocyte chapter, has been subdivided into subsections 3.1- 3.7. Each of the subsections aims to answer particular questions with regards to leucocyte subsets and their role in male subfertility:

3.1- To determine if ASA+ve patients exhibit leucocytospermia and if so, if there is any differential attraction of leucocyte subpopulations and compare them to the ASA-ve patients and controls.

3.2- To determine if microbiologically culture positive patients exhibit leucocytospermia and if so, if there is any differential attraction of leucocyte subpopulations between patient groups or in specific infections.

3.3- To determine if oligospermic patients exhibit leucocytospermia and to analyse the effect of seminal leucocyte subpopulations on various sperm parameters (count, motility, morphology and migration test) with particular emphasis on the sperm count.

3.4- To determine whether asthenospermic patients exhibit leucocytospermia and if there is a relationship between various leucocyte subpopulations and sperm motility.

3.5- To determine if oligoasthenospermic patients exhibit leucocytospermia and to show the effect of various leucocyte subpopulations on sperm parameters with particular emphasis on sperm count and motility.

3.6- To determine if azoospermic patients exhibit leucocytospermia and to which and at what levels the various leucocyte subpopulations are present in azoospermia.

3.7- To determine if poor fertilisers exhibit leucocytopsermia and the true effect of T and B cell subpopulations on the fertilisation rates in an IVF cycle.

Chapter 4, the cytokine chapter, has been divided into subsections 4.1-4.4. Each of the subsections aims to answer particular questions with regards to cytokines and their role in male subfertility:

4.1- To determine if there is an elevation of cytokines in the ASA+ve patients and whether cytokine mediated sperm damage could explain the subfertility in this group of patients.

4.2- To determine if microbilogically culture positive patients exhibit elevated levels of cytokines and if so whether this could explain their subfertility due to cytokine mediated sperm damage.

4.3- To determime the presence of a repertoire of seminal cytokines IL-6, IL-8, IL-10, IL-11, IL-12, TNF-α, and IFN-γ and to evaluate their role in male subfertility and identify any possible networks that may exist.

4.4- To determine the presence of a network of cytokines within the seminal plasma and whether this network may influence fertilisation rates either directly or indirectly.

Chapter 5, the combined leucocyte and cytokine chapter, had the aim to confirm the presence of cell mediated response in the seminal plasma and to identify any correlations between the different leucocyte subpopulations and the individual cytokines and their combined effect on sperm parameters.

Chapter 6, the concluding chapter, draws on all aspects of the study including limitations, future projects and final summary conclusions.

To my knowledge, no previous studies of a similar nature have been carried out.

CHAPTER 2: MATERIALS AND METHODS

2.1 ETHICAL APPROVAL

A literature review was conducted to ensure that the research had not been performed previously and the relevant patient information sheets and consent forms were written (Appendices 1 and 2; pages 249 & 251 respectively). A formal application to the Local Research Ethics Committee (LREC 1998/023) based at Liverpool Women's Hospital was submitted. Ethical approval for the project was subsequently granted and patients were recruited to the study. Patients were recruited at the Liverpool Women's Hospital from July 1999 to July 2001. Patients were asked first verbally if they would be interested in participating in such a study. It was emphasised that this was a voluntary study and their participation did not affect their treatment or success rate of an IVF programme. They were then given the patient information sheet (Appendix 1) which contained further information with regards to the purpose of the study, how will the study be conducted, who will conduct their study, and the confidentiality with regards to the findings of the study. Once the information was read, understood, all questions answered in person, a prospective written informed consent was obtained from all patients participating in the study, using the consent form in Appendix 2.

2.2 GROUP 1: MEN ATTENDING THE SUBFERTILITY CLINICS

Semen samples were obtained from men attending the Liverpool Women's Hospital for infertility investigation. Semen was obtained by masturbation after three to five days of sexual abstinence. The samples were collected in a sterile, wide mouthed, non toxic container (Sterilin 60ml container, WA products, Essex) and processed in the laboratory within 1 hour of ejaculation. Samples did not get exposed to extremes of temperature.

They did not come in contact with either lubricants or latex products. All the samples underwent semen analysis in the laboratory in accordance with the 1999 WHO laboratory manual[13] for the examination of human semen and the study was performed using these reference values. Men were divided into the different subgroups according to the results of two consecutive semen analyses six weeks apart. Given the wide variability between ejaculates, two samples were taken to confidently define an individual's phenotype. The seminology laboratory at the hospital carried out standard seminal analysis on each sample, including details on sperm number, motility, and the presence of bound antisperm antibodies, as detailed by WHO[13]. A sperm migration test was also performed on these samples[185]. The anti-sperm antibodies were detected by the sperm MAR test and evaluated the presence of both IgG and IgA. Parameters obtained from the semen analaysis report are shown in Appendix 3. These men had no other illnesses nor did they take any medication.

Sperm migration test[186]: A 50μl aliquot of liquefied semen was required for each test. It was taken from a well mixed ejaculate specimen at 30 minutes after ejaculation. The microscope objective fields were calibrated so that the numbers of spermatozoa per field could be converted to numbers per unit area. A BEEM® capsule was used as a semen reservoir with a hole in the capsule's cap to take the capillary tube. The semen was placed carefully in the bottom of the reservoir which was held upright in a rack and the cap then closed. The open end of the hylauronate capillary tube was inserted into the semen reservoir so its open end was immersed in the semen. After a period of 60 minutes of incubation at 37°C the capillary tube was removed from the sperm reservoir, wiped clean of any residual semen at its open end and then transferred to a slide for counting. The depth and degree of sperm penetration into the hyaluronate column was assessed microscopically using a magnification of 200-250X. The number of spermatozoa present was counted in each of two microscopic fields at

each of the upper and lower glass faces of the tube at each of the mm distances (e.g. 10mm, 20mm etc.).

The average number of spermatozoa present per field at each distance along the hyaluronate column was calculated and then corrected to give the number per unit area using the known microscope field area. A log-linear regression using millimetre distances along the hyaluronate capillary as the x-axis values and the log transformed numbers of spermatozoa as the y-axis was performed. The vanguard sperm migration distance was calculated[187]. A normal semen sample shows a vanguard migration distance of at least 20mm[188].

Papanicoloau staining technique was used to assess sperm morphology as it is the recommended technique according to the WHO manual[83]. This technique allows good staining of the spermatozoa and the other cells. Also this technique enables one to study the acrosomal and post acrosomal areas of the head, cytoplasmic droplet; mid piece and tail areas well as all these areas are amenable to the stain. The assessment of sperm morphology and migration distance was carried out by the laboratory technician in the Assisted Conception Unit at Liverpool Womens Hospital in July 2001.

Sperm motility is divided into four different grades-

- **Grade 4**: Sperm with progressive motility. These are the strongest and swim fast in a straight line. This is equivalent to WHO motility grade **a**.
- **Grade 3**: (Non-linear motility): These also move forward but tend to travel in a curved or crooked motion. This is equivalent to WHO motility grade **b**.

- **Grade 2**: These have non-progressive motility because they do not move forward despite the fact that they move their tails. This is equivalent to WHO motility grade **c.**
- **Grade 1**: These are immotile and fail to move at all. This is equivalent to WHO motility grade **d.**

All the samples underwent testing for genito-urinary infections such as *Ureaplasma, Mycoplasma, Chlamydia trachomatis, Trichomonas vaginalis* and other organisms such as *Enterococcus, Streptococcus* etc. The swabs were taken directly from semen and sent to the microbiology laboratory for culture. The samples were transported to the Royal Liverpool University Hospital and processed with 24 hours to avoid being frozen to -70°C.

Direct culture of semen is ideally the best practice method to detect male genital tract infection as it is 100% specific. However, these are technically demanding, needing cold chain particularly for *Chlamydia trachomatis* to preserve specimen viability in transport and are not readily available. Furthermore, the results can take up to eight days. However, these tests were done almost nine years ago and now Nucleic Acid Amplification Tests (NAAT) may be more applicable as they are both highly sensitive and specific (95%)[189]. The results of NAAT are received within a few hours. Thus, NAAT is replacing culture as the tests of choice in detecting male genital tract infections. However, to date there are no commercial NAAT tests available.

After routine semen analysis, samples were centrifuged at 300g for 10 minutes and the supernatant removed and stored at -20°C until assayed for cytokines as described in section 2.6.2 to prevent the effect of proteases on the individual cytokines. The samples were assayed in the same run to reduce inter assay variability. The remainder of the sample underwent

immunocytochemical staining, the established method for accurately detecting leucocyte subpopulations[102].

2.3 GROUP 2: PARTNERS OF WOMEN ATTENDING THE IVF CYCLE

An embryologist prepared the fresh sperm sample for IVF and the remainder of the sample was then used for the analysis of cytokines. None of the samples were discarded. Different grading schemes are used for embryos when they reach the blastocyst stage (around day three of their development). For this study an in house grading system of Grades 1-4 was used. Although grading systems vary slightly from one unit to another, they all produce similar results.

The embryo grading used the following criteria as shown in Figure 2.2 [190]:

- The number of cells present;
- How fast the cells are dividing;
- Whether the division is even;
- Whether there are any fragments of cells present.

The embryo is of high quality if it has an optimal cell number, the cells are fairly regular in size, even cell division and there is no fragmentation and is thus referred to as Grade 1. If the embryo has even cell division and small fragmentation then it is Grade 2. If the embryo is moderately fragmented and has unevenly sized cells (blastomeres) then it is Grade 3. This embryo is expected to have a significantly lower chance of implantation. If the embryo is severely fragmented and has unevenly sized cells then it is Grade 4. This embryo probably does not have much chance to implant and become a viable pregnancy.

Most of the published embryo grading studies are small and generally from single centres, and likely to be subjective and potentially vary between operators and laboratories. Thus the Association of Clinical Embryologists in conjuction with the British Fertility Soceity came up with a cleavage stage embryo grading system in 2008, based on blastomere number, blastomere size and fragmentation[191].

2.4 PREPARATION OF SEMINAL LEUCOCYTES

Preparation of the slides:

Details of preparation of the slides are shown in Appendix 4.

Semen samples were diluted to 10ml with phosphate buffered saline (PBS), and washed by centrifugation for 10 minutes at 300g at room temperature. The clear supernatant was discarded. The final cellular pellet was resuspended in 2ml of PBS/BSA (containing 0.1% BSA w/v). An aliquot of this suspension was mixed 1:1 in Trypan blue (which stains non-viable cells). Using a haemocytometer, the cellular concentration and percentage viability was assessed. By altering the dilution factor of the suspension, the total round cell concentration (including non-viable cells) was adjusted to 1 x 10^6/ml in PBS/BSA. The round cells were leucocytes and germ cells.

Constantly mixing to ensure an even suspension of cells, 50μl was applied to each spot on Teflon-coated slides. Slides were air dried overnight, before fixing for 10 minutes in acetone. Wrapped in foil, slides were stored at -20°C until staining.

2.5 IMMUNOHISTOCYTOCHEMISTRY

World Health Organisation recommends peroxidase staining as the standard method and immunocytochemistry using monoclonal antibodies as the gold standard for the detection of

leucocytes. Although the sensitivity of peroxidase test for the detection of leucocytospermia currently could be considered low[192], at the time of this study no commercial flow cytometry methods were approved. Immunohistological staining employing monoclonal antibodies against all specific WBC subpopulations is expensive, time consuming and not standardised[193].

In the past 10 years, flow cytometry technology has been used for sperm analysis[194]. One study has shown that flow cytometry using monoclonal antibodies is a simple and reproducible method for detecting semen leukocytes and that the correlation between peroxidase test and flow cytometry results is good but not absolute[195]. This technique, when used alongside monoclonal antibodies, allows a rapid, multiparameter analysis of particles and is suitable for counting and typing seminal leucocytes.

Mouse IgG (Coulter, Luton, UK) in a 1:100 dilution was used as a control for the immuno-histochemistry as it has no specificity.

2.5.1 Staining of seminal leucocytes

Materials required:

-Primary monoclonal antibodies as required (Dako Ltd. High Wycombe, UK.)

-BSA

-Rabbit anti-mouse IgG (Dako Ltd. High Wycombe, UK - Code Z 259)

-Normal human serum (NHS), heat inactivated

-Alkaline phosphatase: anti-alkaline phosphatase complex (APAAP, Dako Ltd. High Wycombe, UK - Code D651)

-Fast Red TR salt (Sigma-Aldrich. Gillingham, UK)

-Haemalum stain (BDH. Poole, UK)

-0.45μm filter

-Humidified slide box

-Dako pen

-Aquamont (BDH. Poole, UK)

-Tris buffered saline (TBS);

Tris 50mM	6.0g/l
NaCl 150mM	8.7g/l

Made up to 1 litre with distilled water, and adjusted to pH 7.6 using HCl

-Alkaline phosphatase substrate:

Napthol AX-MX phosphate (Sigma)	10mg
Dimethyl formamide	1.0ml
0.1 M Tris buffer pH8.2	50mls
Levamisole 1.0M solution	50μl

Stock solution prepared in a glass bottle and stored at 4°C.

Slides were removed from the freezer and allowed to reach room temperature. Areas to be stained were marked with a Dako pen to separate adjacent wells. The primary monoclonal antibodies were diluted to the working ratios as shown in Table 2.2 in tris buffer saline (TBS) and 0.5% BSA (working dilution is the amount necessary for binding to all target antigens, without non-specific binding). 50μl of each antibody was added to wells, and incubated for 30 minutes at room temperature, in a humidified chamber (to prevent drying of the samples). Slides were rinsed in TBS and then washed twice for five minutes in TBS. Rabbit anti-mouse immunoglobulin (Dako, High Wycombe, UK) was diluted 1:25 in TBS and 5% NHS, and 50μl of this was added to each well, and incubated as above for 30 minutes. Following this,

the rinse and two washes in TBS were repeated. Alkaline phosphatase: anti-alkaline phosphatase complex (APAAP) (Dako, High Wycombe, UK) was diluted 1:50 in TBS and added 50 μl to each well and incubated for 30 minutes. The rinse and two washes in TBS were repeated. Antibody binding was enhanced by repeating the addition of Rabbit anti-mouse immunoglobulin and APAAP solutions (with intervening washes), incubating for just 10 minutes each time. Slides were washed twice in TBS, then incubated for 20 minutes with filtered Alkaline Phosphatase substrate and Fast Red (50 μl per well). Slides were washed once in TBS then once in distilled water, before counterstaining with Haemalum (BDH. Poole, UK) for 15 seconds (stains nuclei blue). Slides were washed thoroughly in tap water and mounted in Aquamount (BDH. Poole, UK).

2.5.2 Antibodies used in the study

The following monoclonal antibodies were used in the study as shown in Table 2.1.

2.5.3 Counting of Leucocytes

The number of positively stained (red-pink) leucocytes for each antibody were counted in ten high power fields (HPF = x320 magnification) using a light microscope fitted with a graticule. Mean values of the ten counts were calculated, and used for an average value per HPF. This method of quantification of leucocyte subpopulations was achieved using original sperm concentration, according to the method of Wolff and Anderson 1988[94]. A manual count was performed as there were no standardised instrument methods at the time when the study was performed. Ten high power fields were used as the average counting method for leucocytes as suggested by published studies at the time[94;98;196]. The specificity of each antibody (the type of leucocyte it binds to, resulting in red-pink staining) is given in Table 2.1. CD45 binds to a common leucocyte antigen, thus staining all leucocytes.

Table 2.1: Details of the monoclonal antibodies used in this study (antigenic markers: CD-cluster of differentiation, Ig-immunoglobulin).

ANTIBODY to	SPECIFICITY	DILUTION	CLONE Obtained from
CD3	All T-lymphocytes	1:100	UCHT1; Serotec, Oxon, UK.
CD4	Helper T-lymphocytes	1:50	MT310; Dako, High Wycombe,UK
CD8	Cytotoxic T-lymphocyte	1:50	DK25; Dako, High Wycombe,UK
CD14 (supernatant)	Monocytes/macrophages	Neat	3C10; Cell, Canton, MA USA
CD16 (supernatant)	Granulocytes, natural killer cells	1:50	3G8; Cell Canton, MA USA
CD 20	B-Lymphocytes	1:50	4KB128; Dako, High Wycombe,UK
CD45 (supernatant)	All leucocytes (leucocyte common antigen)	Neat	F10-89-4; Cell Canton, MA USA
CD69	ActivatedT-lymphocytes, B-lymphocytes and macrophages	1:20	CH/4; Serotec Oxon, UK
MHC class II supernatant antigens	MHC class II, macrophages	Neat	L243; Cell Canton, MA USA

In wells incubated with CD45, both positive and negative (unstained, identified by blue nucleus) cells were counted. This proportion was used to determine the proportion of leucocytes relative to other round cells (immature germ cells) in the ejaculate. The percentage of CD45 positive cells were determined from the number of CD45 positive cells divided by the sum of both the CD45 positive and negative cells. Since all samples were initially diluted to 1x 10^6/ml, these results represent the total number of leucocytes per ejaculate. The absolute counts of the monoclonal antibodies were used for analysis as some of the monoclonal antibodies can cross react with other cells and hence the medians were used for the analysis rather than expressing them as a percentage of the total round cell count.

2.5.4 ASA Study

The seminology laboratory at the hospital carried out a number of tests on each sample, including details on sperm number, motility and the presence of bound antisperm antibodies, as detailed by WHO and discussed by Biljan et al[185]. The samples were divided into two groups (ASA+ve, ASA–ve) on the basis of bound ASA levels above 50% as detected by the MAR test (WHO, 1992). After routine semen analysis, the remainder of the sample underwent immunocytochemical staining.

2.5.5 Statistical Analysis

Statistical analysis of results was performed using SPSS version 13 software package. Using the Shapiro Wilkes test for non normality it was established that the results obtained were not normally distributed. This indicated that a non parametric test should be used. The Mann-Whitney U test was applied to analyse the difference between cell counts from ASA-ve / ASA+ve / control groups of patients and differences between sperm parameters. Spearman's rank correlation coefficient was used to determine the correlation between leucocyte count

and sperm parameters for both the ASA-ve and ASA+ve groups of patients. Similarly, the Mann-Whitney U test was applied to analyse the difference between cell counts from culture positive and culture negative groups of patients and differences between sperm parameters. Spearman's rank correlation coefficient was used to determine the correlation between leucocyte count and sperm parameters for culture -ve and culture +ve patients. Statistical significance was taken at the 5% confidence level ($p<0.05$) and 1% confidence level ($p<0.01$). The numbers of patients were relatively small as this was aimed as a pilot study and if significant levels of leucocytes were found then an adequately powered study would be required to determine the statistical and the clinical significance of these findings. Thus both levels of statistical significance were looked at as these numbers were on the low side and 1% confidence level would make this study more robust.

2.6 ELISA

2.6.1 Preparation and storage of the semen samples

The supernatant was removed from the previously centrifuged samples and stored at -20°C until assayed.

2.6.2 Cytokines assays

Cytokine sandwich ELISAs are sensitive enzyme immunoassays that can specifically detect and quantitate the concentration of soluble cytokine and chemokine proteins. The basic cytokine sandwich ELISA method makes use of highly-purified anti-cytokine antibodies (capture antibodies) which are noncovalently adsorbed ("coated" – primarily as a result of hydrophobic interactions) onto plastic microwell plates. After plate washings, the immobilised antibodies serve to specifically capture soluble cytokine proteins present in samples which are applied to the plate.

After washing away unbound material, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labeled avidin or streptavidin stage. Following the addition of a chromogenic substrate-containing solution, the level of a coloured product generated by the bound, enzyme-linked detection reagents can be conveniently measured spectrophotometrically using an ELISA plate reader at an appropriate optical density (OD). Data storage and reanalysis are greatly simplified when the plate reader is connected to a computer. By including serial dilutions of a standard cytokine protein solution of known concentration, the sandwich ELISA supports the development of standard curves.

Standard curves (aka "calibration curves") are generally plotted as the standard cytokine protein concentration (typically ng or pg of cytokine/ml) versus the corresponding mean OD value of replicates. The concentrations of the putative cytokine-containing samples can be interpolated from the standard curve.

Cytokine concentrations were measured using specific commercial ELISA kits (TNF-α by Genzyme Duoset kit Cambridge, Massachusetts, USA and IL-6, IL-8, IL-10, IL-11, IL-12 and IFN-γ from R&D Systems, Abingdon, U.K). The range of measurement of the cytokines was IL-6 (3.125-300 pg/ml), IL-8 (31.2-2000 pg/ml), IL-10 (31.25-2000 pg/ml), IL-11 (15.6-1000 pg/ml), IL-12 (7.8-500 pg/ml), IFN-γ, TNF-α (15.6-1000 pg/ml). The sensitivity of the assays for IL-6, IL-8, IL-10, IL-11, IL-12, IFN-γ and TNF-α were less than 0.6pg/ml, 2.6pg/ml, 6.3pg/ml, 4.4pg/ml, 2pg/ml, 8pg/ml and 1.6pg/ml respectively.

An example of how an assay was performed is described below.

ELISA for Human IL-8

Required:

1. The following reagents from R&D systems:

   Capture ab Mouse anti-human IL-8 (cat no. MAB208).

   Second ab Goat anti-human IL-8, biotinylated (cat no. BAF208).

   Detection agent Streptavidin horseradish peroxidise (Zymed cat no. 43-4323).

   Standard Recombinant human IL-8 (cat no. 208-IL-010).

2. Substrate: Sigma one-step TMB/peroxide, part # T-8540.
3. BSA: need fatty acid-free, suitable for ELISA, e.g. Sigma part # A-3803.
4. Microtiter plates, Nunc MaxiSorp part # 439454.
5. 37°C incubator, plate reader, polypropyplene tubes.
6. Stop solution: 2N H_2SO_4.
7. Coating solution: PBS.
8. Blocking buffer: PBS + 1% BSA+ 5% sucrose + 0.05% sodium azide.
9. Wash buffer: PBS + 0.05% Tween 20.
10. Antibody diluent (used for biotinylated 2nd ab and streptavidin-HRP reagent): PBS + 0.05% Tween 20 + 0.1% BSA.

Preparation of reagents and buffers:

Capture antibody: Mouse anti-human IL-8, Stock =500 µg/ml in PBS.

Coat at 4µg/ml i.e. 1:125 dilution in PBS.

For one plate will require 80µl in 10 mls.

IL-8 Standards: Use polypropylene tubes, dilute in culture medium without FBS if testing culture supernatants. Stock=10µg/ml, reconstituted in 1.0 ml PBS+0.1% BSA. Aliquots frozen at -20°C, 50µl per tube.

Dilute stock 5µl +10mls medium = 5000pg/ml

Then prepare serial doubling dilutions i.e. 300µl + 300µl medium to produce the following standards: 2500, 1250, 625, 312.5, 156, 78.

Also require medium control and medium + FBS control.

Secondary antibody: Goat anti-human IL-8 biotinylated, 50µg/ml stock in PBS +0.1% BSA.

Use at 0.02µg/ml ie 1:2500 in PBS/Tw/BSA.

For one plate will require 4µl/10 mls.

Streptavidin-HRP: Dilute 1:4000 in PBS/Tw/BSA; prepare 1:400 dilution first (can store at 4°C), then 1:10.

Method: The plates were coated with 100µl per well of capture antibody. They were subsequently incubated at room temperature overnight. The wells were washed the following day five times with PBS/Tween. It is very important to fill wells forcefully and decant or blot to remove all the liquid. Each well was coated with 300µl blocking buffer and kept for 90 minutes at room temperature. Meanwhile the standards and the samples were prepared. The wells were washed again with PBS/Tween. 100µl of standards and samples were added to the wells. It was covered and incubated for 1 hour. The wells were washed five times again with PBS. 100µl of the 2^{nd} ab was added to each well. It was covered and incubated at 37°C for 1 hour. The wells were washed five times with PBS. 100µl of Streptavidin-HRP (1:4000) was added to each of the wells. It was covered and incubated for 20 minutes at room temperature. Subsequntly, the wells were washed five times again with PBS. 100µl of substrate TMB/H_2O_2 was added to each well. The plates were then incubated for 20 minutes at room temperature in the dark. 100µl of stop solution was added per well. The plates were read at OD 450nm and 550nm within 60 minutes. The 550 readings were substracted from 450. The assays for the remaining cytokines are the same except for different capture antibodies.

An example of how a calibration curve looks is depicted in the Figure 2.2.

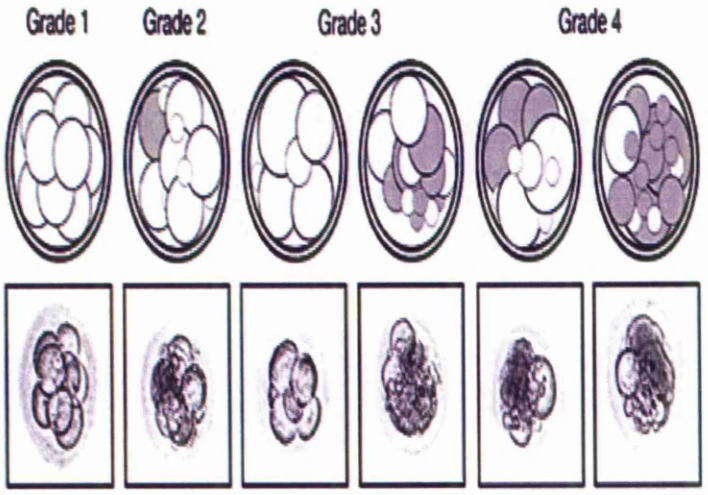


Figure 2.1: Embryo grading (Grade 1- no fragmentation, Grade 2- <30% fragmentation, Grade 3- 30-50% fragmentation, Grade 4- > 50% fragmentation)[190]. Reprinted with permission from An Atlas of Human Gametes and Conceptuses, 2001; Informa Healthcare.

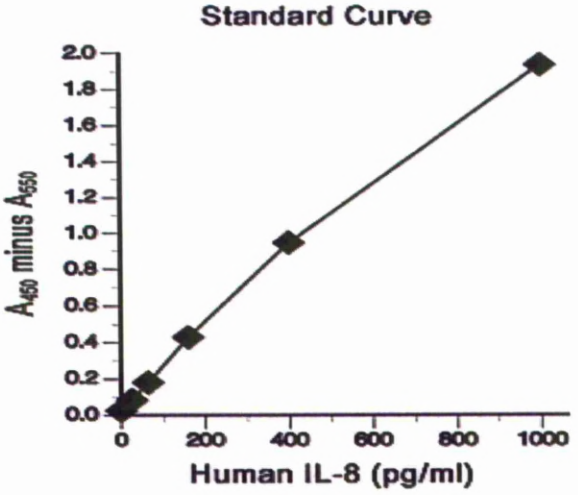


Figure 2.2: Cytokine calibration curve for human IL-8 using ELISA kit and calibrated to National Institute of Biological Standards and Control. Reprinted with permission from Guidebook to cytokines and their receptors; 1994, Oxford University Press.

2.6.3 Statistical Analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS) software version 13. The Shapiro Wilkes test was used to test for normal distribution. The individual cytokine levels in the normospermic group were compared to the levels of the respective individual cytokine from the other subsets. As the data was not normally distributed, statistical analysis was performed using the Mann-Whitney non-parametric two-sample test. Statistical analysis was performed using the Mann-Whitney test to compare poor fertilisers and good fertilisers. Correlations between cytokine concentrations were tested using the non-parametric Spearman's correlation test. Similarly, using the Spearman's correlation coefficient, correlations between cytokine concentrations and the fertilisation rates were measured. The correlation between the semen parameters and fertilisation rates was also measured using Spearman's correlation coefficient.

There was a lack of published data in this field, at the time of recruitment, making it difficult to estimate accurate numbers for this study. However, a power calculation was performed with the assistance of a statistician. The incidence of leucocytospermia was quoted to be approximately 10% in infertile men. Based on this incidence, a sample size of 80 was estimated with 10 patients in each group of subfertile men. The numbers of patients were relatively small as this was aimed as a pilot study and if significant levels of cytokines were found then an adequately powered study would be required to determine the statistical and the clinical significance of these findings. Thus both levels of statistical significance were looked at as these numbers were on the low side and the 1% confidence level would make this study more robust.

CHAPTER 3: LEUCOCYTE SUBSETS IN DIFFERENT INFERTILE MALE GROUPS AND IN FERTILE CONTROLS

The relationship between leucocytes and the fertilising potential of an ejaculate remains highly controversial. Leucocytopsermia, a frequent finding in infertile patients, is often associated with poor semen quality. However, some authors have failed to show a robust association between sperm and leucocytospermia and these have been discussed in Chapter 1. Most of the previously performed studies investigated the effects of leucocytes on sperm motility. However, it is still unclear whether leucocytospermia actually has any effect on fertilisation or not.

Hence, the aim was to investigate not alone the role of leucocytospermia in male subfertility but also to look at what effect the different leucocyte subpopulations have on various sperm parameters such as motility, numbers and fertilisation potential. The leucocyte populations studied and characterised were the T lymphocytes, B lymphocytes, monocytes / macrophages, activated T and B lymphocytes and macrophages, granulocytes and MHC class II macrophages.

This chapter has been subdivided into subsections 3.1- 3.7. Each of the subsection aims to answer particular questions with regards to leucocyte subsets and their role in male subfertility:

3.1- To determine if ASA+ve patients exhibit leucocytospermia and if so, if there is any differential attraction of leucocyte subpopulations and compare them to the ASA-ve patients and controls.

3.2- To determine if microbiologically culture positive patients exhibit leucocytospermia and if so, if there is any differential attraction of leucocyte subpopulations between patient groups or in specific infections.

3.3- To determine if oligospermic patients exhibit leucocytospermia and to analyse the effect of seminal leucocyte subpopulations on various sperm parameters (count, motility, morphology and migration test) with particular emphasis on the sperm count.

3.4- To determine whether asthenospermic patients exhibit leucocytospermia and if there is a relationship between various leucocyte subpopulations and sperm motility.

3.5- To determine if oligoasthenospermic patients exhibit leucocytospermia and to show the effect of various leucocyte subpopulations on sperm parameters with particular emphasis on sperm count and motility.

3.6- To determine if azoospermic patients exhibit leucocytospermia and to which and at what levels the various leucocyte subpopulations are present in azoospermia.

3.7- To determine if poor fertilisers exhibit leucocytopsermia and the true effect of T and B cell subpopulations on the fertilisation rates in an IVF cycle.

3.1 STUDY NUMBER 1: Leucocyte subsets in Antisperm Antibody Positive versus Antisperm Antibody Negative patients versus the Controls

3.1.1 Introduction

Autoimmunity is a condition in which the immune system is sensitised to self tissues. Spermatozoa contain at least three powerful membrane antigens, against which a specific immune response may be mounted via the production of antibodies which may be formed as a result of local sequestration of leucocytes[197]. The role of cell mediated immunity (and hence the significance of leucocytospermia) in the production of ASA is much debated. A slight but

not statistically significant correlation between ASA and leucocytospermia has been reported[82], whereas in another study the presence and levels of ASA were completely unrelated to the concentration of leucocytes.

Various subgroups of leucocytes play different roles in autoimmunity, which may account for their varying incidence amongst ejaculate with antisperm antibodies. Antibodies are produced by B lymphocytes. γδ T cells are present in the human semen, they comprise about one third to one half of the total number of T lymphocytes in this compartment and their mean concentration is increased about ten fold in men whose sperm contains bound auto-antibodies[28]. This suggests that a localised autoimmune response to sperm is associated with activation and proliferation of γδ T cells in the male reproductive tract[28].

The hypothesis was that there would be a difference in the leucocyte populations found in ASA positive and ASA negative patients which might reflect the different aetiological roles of leucocytes in these two cases. Therefore the aim was to examine the leucocyte subpopulations in the semen and to compare these between the antibody positive, antibody negative subjects and control subjects.

3.1.2 Materials and Methods

MAR test was used to detect bound ASA levels as outlined in Chapter 2 section 2.5.4.

3.1.3 Statistical Analysis

As outlined in Chapter 2 section 2.5.5.

3.1.4 Results

64 patients were recruited into this study. 26 fertile men were recruited as the control group. These 26 men were attending the IVF clinic due to female factor infertility, were ASA-ve and their partners had achieved a pregnancy and thus were considered to be an adequate control group. Twenty of the azoospermic men were removed from the analysis as there was no sperm to bind to the ASA. Seventeen patients were found to be ASA+ve. Twenty one patients were ASA-ve. Eighteen men had equivocal results (level of binding < 50%) and thus not considered into further analysis. The sperm parameters (sperm count, motility, morphology and sperm migration test) were significantly reduced in the ASA+ve group when compared to the normal control group ($p<0.01$). The morphology and the SMT were significantly reduced in the ASA+ve group when compared to the ASA-ve group at $p<0.01$ and $p<0.04$ respectively (Table 3.1).

The subpopulations of leucocytes present amongst ASA+ve, ASA-ve and control groups are given in Table 3.2. The total leucocyte number (CD45 +ve cells) was higher among ASA+ve (11.1) than ASA-ve (5.35) samples, although it did not reach statistical significance. The T lymphocytes (CD 2, 3, 4 and 8), B lymphocytes (CD20) and the large granular lymphocyte (CD56) were significantly higher in the ASA+ve (1.3, 0.8, 1.1, 0.8, 0.6 and 0.5) and ASA-ve groups (1.15, 1.05, 1.65, 0.85, 0.75 and 0.5) when compared to the control group (0.15, 0.15, 0.2, 0.1, 0.2, and 0.1) respectively. However, there was no statistically significant difference amongst the T lymphocyte counts between the ASA+ve and ASA-ve groups. The similar finding was mirrored in the analysis of activated T and B cells (CD69).

Table 3.1: Comparison of sperm parameters between ASA negative, positive and control subjects. Values are expressed as medians, with the range for each group given in brackets. P value relates to a statistical significance between ASA negative vs. controls, ASA positive vs. controls and ASA positive vs. negative. *Significant at 5% level **significant at 1% level.

Sperm Parameters	ASA Negative (n=21) Median and Ranges	ASA Positive (n=17) Median and Ranges	Controls (n=26) Median and Ranges	P –value Negative vs. controls	P –value Positive vs. controls	P –value Positive vs. negative
Sperm count 10^6/ml	35.5 (8.5-52.5)	26 (11-40)	104 (73-119)	0.002**	0.0001**	0.83
Motility %	25 (15.5-36.25)	20 (17.5-30)	90 (80-99)	0.0001**	0.0001**	0.46
Morphology % normal forms	30 (19.25-34.25)	12 (10-23.25)	41 (31-48)	0.003**	0.0001**	0.01*
SMT 10^6/ml	4.5 (1.5-10)	1.5 (0.2-4)	24 (15-30)	0.001**	0.0001**	0.04*

Table 3.2: Comparison of leucocyte subpopulations between each subject group. Values are expressed as median cell count (per 10 high power fields), with ranges for each group given in brackets. P value indicates that results reached statistical significance between the following comparisons: ASA Negative vs. controls, ASA positive vs. controls, ASA negative vs. ASA positive. *Significant at 5% level **significant at 1% level.

Antibody To	ASA Negative (n=21) Median and Ranges	ASA Positive (n= 17) Median and Ranges	Controls (n=26) Median and Ranges	P –value Negative vs. controls	P –value Positive vs. controls	P –value Positive vs. negative
CD45	5.35 (3.15-25.15)	11.1 (4.7-20.1)	2.25 (0.63-4.95)	0.01**	0.0001**	0.47
CD2	1.15 (0.55-1.95)	1.3 (0.7-2.1)	0.15 (0-0.55)	0.01**	0.001**	0.61
CD3	1.05 (0.45-1.7)	0.8 (0.4-1.7)	0.15 (0.05-0.65)	0.004**	0.003**	0.93
CD4	1.65 (0.85-2.35)	1.1 (0.8-2.1)	0.2 (0-0.3)	0.001**	0.0001**	0.61
CD8	0.85 (0.45-1.3)	0.8 (0.6-1)	0.1 (0-0.4)	0.01**	0.001**	0.74
CD14	1.85 (0.55-2.9)	1.6 (0.7-2.4)	0.55 (0.1-2.25)	0.07	0.08	0.84
CD16	2.65 (1.5-5.6)	1.7 (0.8-6.8)	0.65 (0.05-1.05)	0.0001**	0.002**	0.49
CD20	0.75 (0.35-1.85)	0.6 (0.4-1.1)	0.2 (0-0.6)	0.01**	0.003**	0.99
CD56	0.5 (0.3-0.75)	0.5 (0.3-0.7)	0.1 (0-0.35)	0.004**	0.01**	0.82
CD69	1.2 (0.35-1.7)	0.9 (0.4-1.7)	0 (0-0.1)	0.0001**	0.0001**	0.72
CD4/CD8 Ratio	1. 9	1. 37	2			

The macrophages and monocytes (CD14) were elevated in the ASA+ve (1.6) and ASA-ve (1.85) groups but this elevation was not statistically significant when compared to the control group (0.55). However, the granulocytes (CD16) were significantly elevated in both the ASA+ve and ASA-ve groups when compared to the control group (Figure 3.1). However, there was no statistically significant elevation between the ASA+ve and the ASA-ve groups. Figure 3.2 represents the median and ranges of activated T and B cells (CD69) in the different groups.

The analyses of the different groups were repeated after excluding the patients who were microbiology culture positive. This was done to find out the true effect of ASA on sperm parameters. This included 12 patients in the ASA negative group, 11 patients in the ASA positive group. The control group (n=26) had negative cultures. Table 3.3 represents the median and ranges of the sperm parameters and the CD counts in the different groups after exclusion of the infected samples. The results mirrored the previous analysis.

There was a good correlation between leucocytopsermia in the ASA+ve group and poor sperm morphology as shown in Figure 3.3. Figures 3.4 - 3.7 illustrate the CD2, CD8, CD45 and CD69 cells respectively in the ASA+ve sample. Figure 3.8 depicts the various correlations identified between the T and B cells in the ASA+ve patients. There was a positive correlation ($r= 0.74$, $p<0.01$) between the T cells (CD3) and B cells (CD20) which is depicted in Figure 3.9.

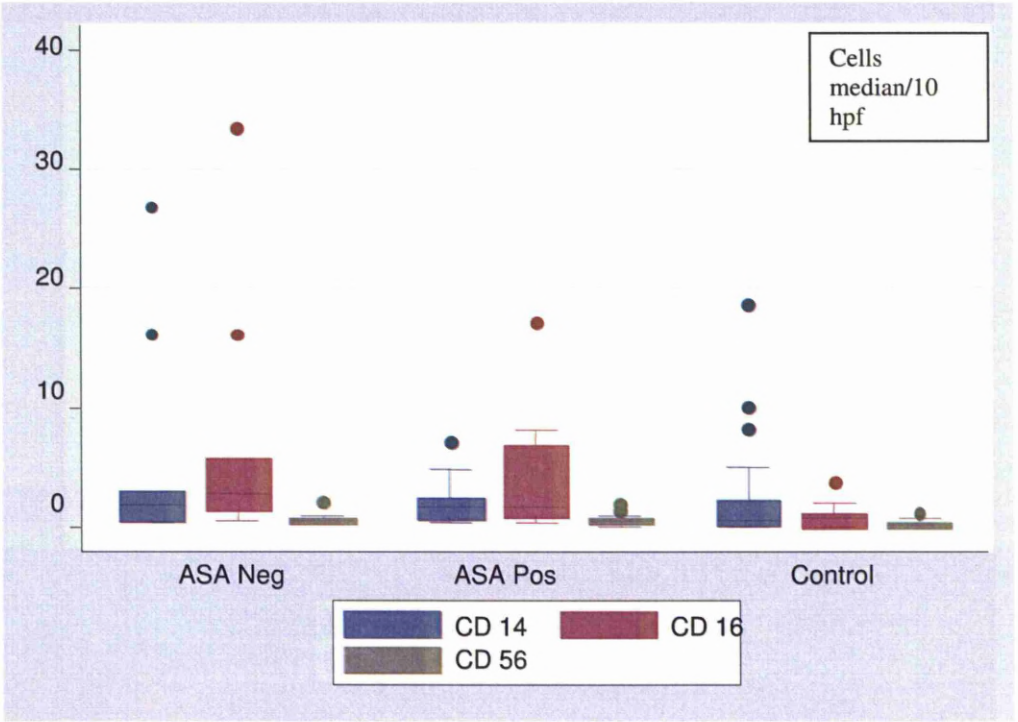


Figure 3.1: Box plots showing macrophages, granulocytes and large granular lymphocytes.

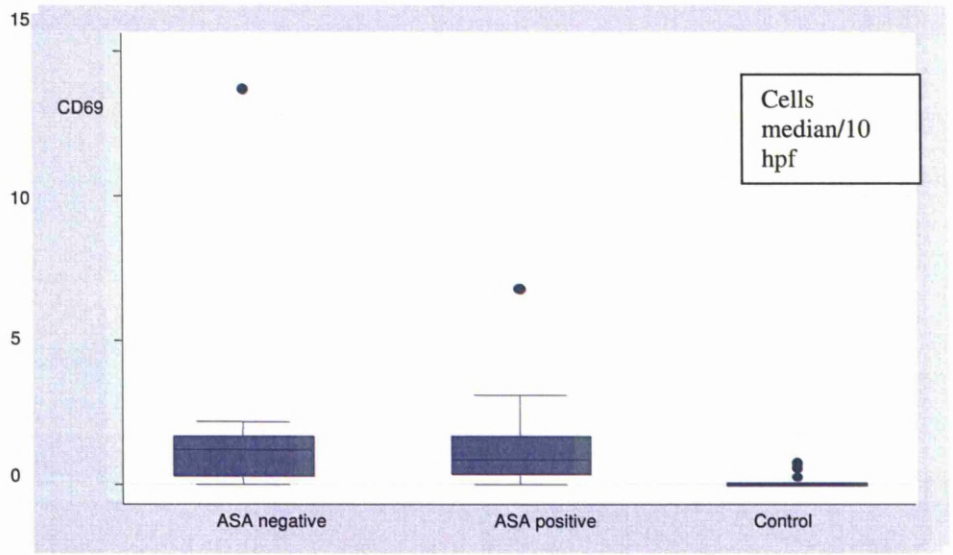


Figure 3.2: Box plot representing CD 69 (activated T and B cells) in the ASA positive, ASA negative and controls

Table 3.3: Median and ranges for the sperm parameters and the CD counts (expressed as cells/10 hpf) for the ASA positive and negative groups vs. controls who are culture negative. *Significant at 5% level **significant at 1% level.

	ASA negative (n=12) Median and ranges	ASA positive (n=11) Median and ranges	Controls (n=26) Median and ranges	Negative vs. controls	Positive vs. controls
CD 45	12.4 (4.3-43.8)	5.9 (3.7-12.1)	2.25 (0.65-4.7)	**0.003****	**0.01****
CD 2	1.7 (0.2-2.2)	1.8 (0.7-2.2)	0.15 (0-0.55)	**0.04***	**0.001****
CD 3	1.3 (0.4-1.9)	0.8 (0.4-2.2)	0.15 (0.05-0.65)	**0.02***	**0.01****
CD 4	1.8 (0.6-3.6)	1 (0.8-2.1)	0.2 (0-0.3)	**0.003****	**0.001****
CD 8	0.9 (0.2-2.1)	0.9 (0.6-1)	0.1 (0-0.4)	0.1	**0.003****
CD 14	2.8 (0.5-16.2)	2 (0.7-2.8)	0.55 (0.1-2.25)	0.06	0.08
CD 16	3 (1.5-16.2)	1.7 (0.8-7.4)	0.65 (0.05-1.05)	**0.006****	**0.01****
CD 20	0.9 (0.3-3)	0.5 (0.4-1.7)	0.2 (0-0.6)	**0.01****	**0.01****
CD 56	0.5 (0.3-0.9)	0.5 (0.3-0.8)	0.1 (0-0.35)	**0.01****	**0.01****
CD 69	1.7 (0.2-2.2)	0.8 (0.4-1.7)	0 (0-0.1)	**0.006****	**0.0001****
L 243	3.9 (1.7-15.6)	2.2 (0.9-3.8)	0.7 (0.3-1.8)	**0.01****	**0.03***
CD4 / CD8 Ratio	2	1.11	2		

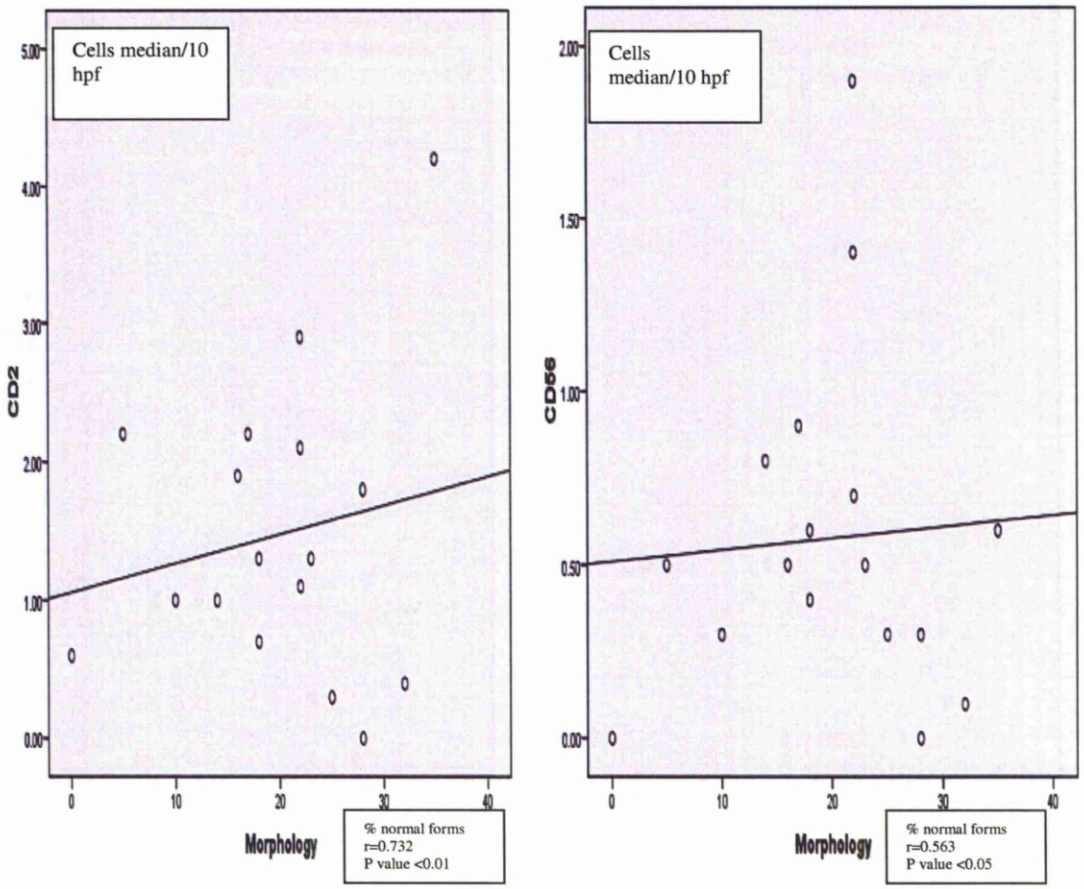


Figure 3.3: Correlations between leucocytes with sperm morphology in the ASA+ve group.

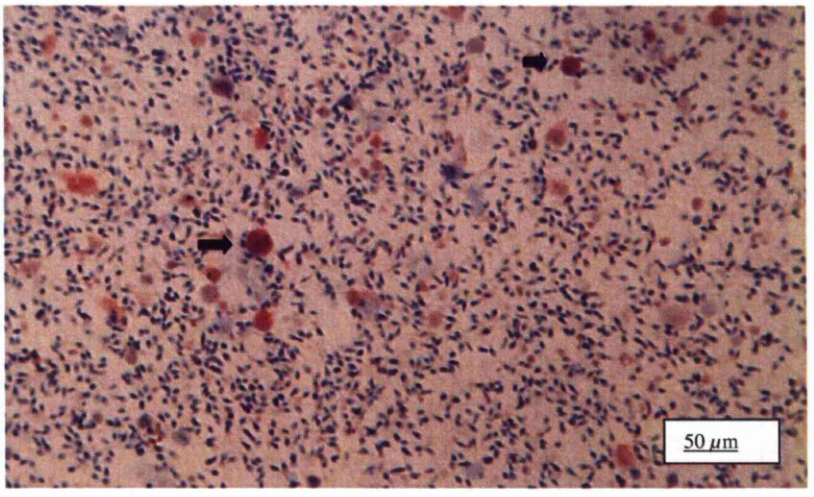


Figure 3.4: CD 2 cells highlighted in the ASA+ve sample.

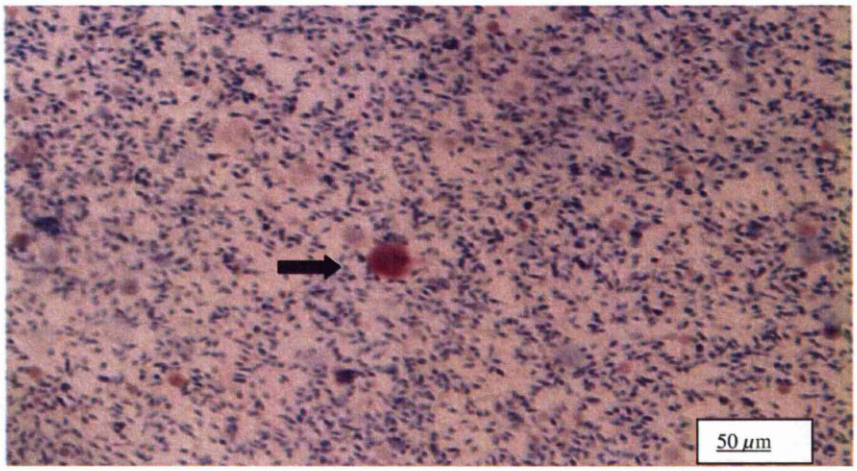


Figure 3.5: CD 8 cell highlighted in the ASA+ve sample.

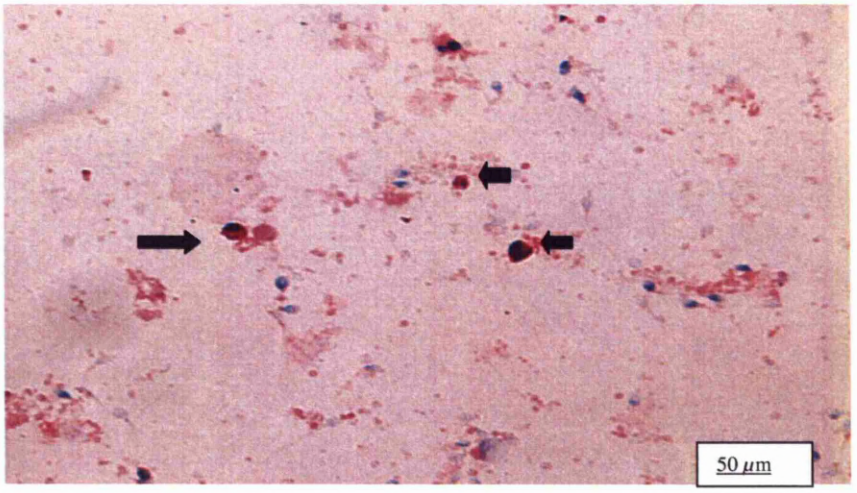


Figure 3.6: CD 45 cells present in the ASA+ve sample.

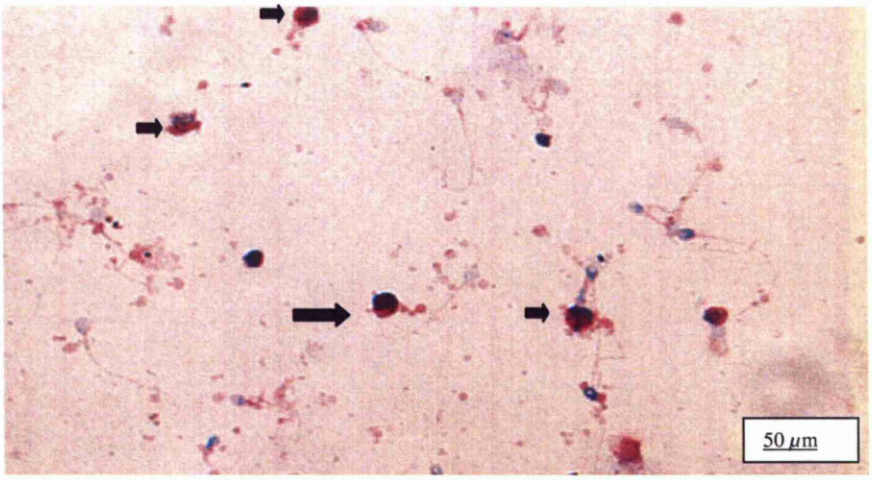
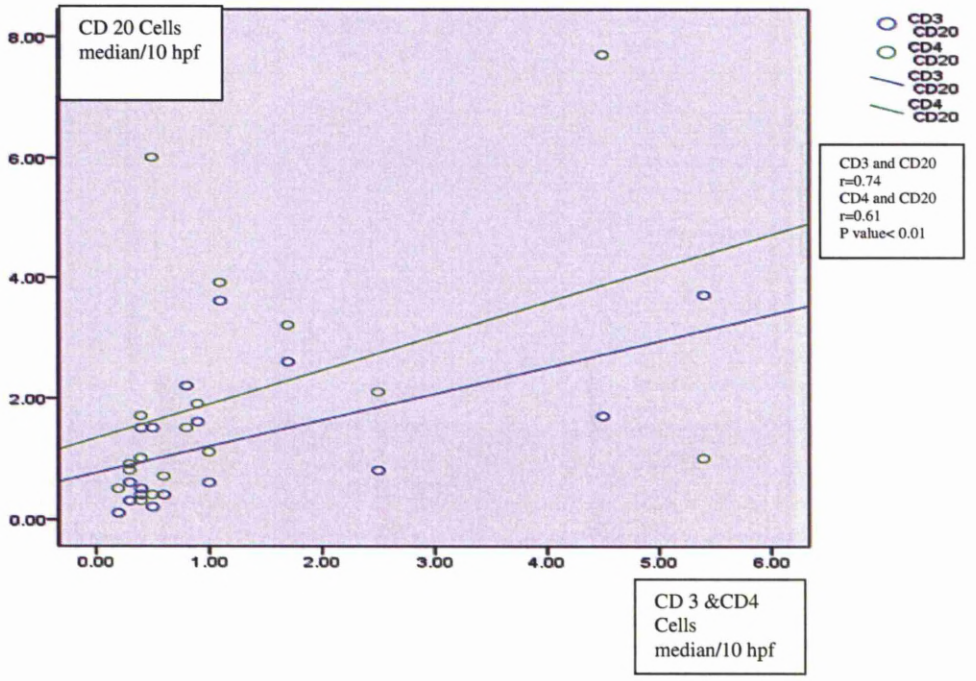


Figure 3.7: CD 69 cells in the ASA+ve sample.



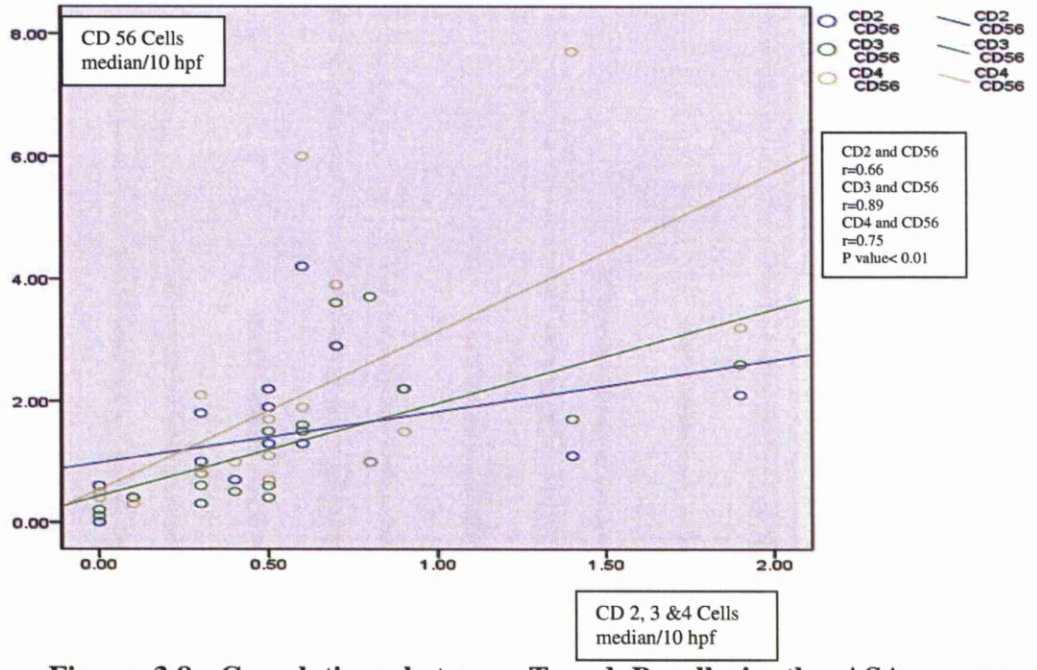


Figure 3.8: Correlations between T and B cells in the ASA+ve group (r = correlation coefficient).

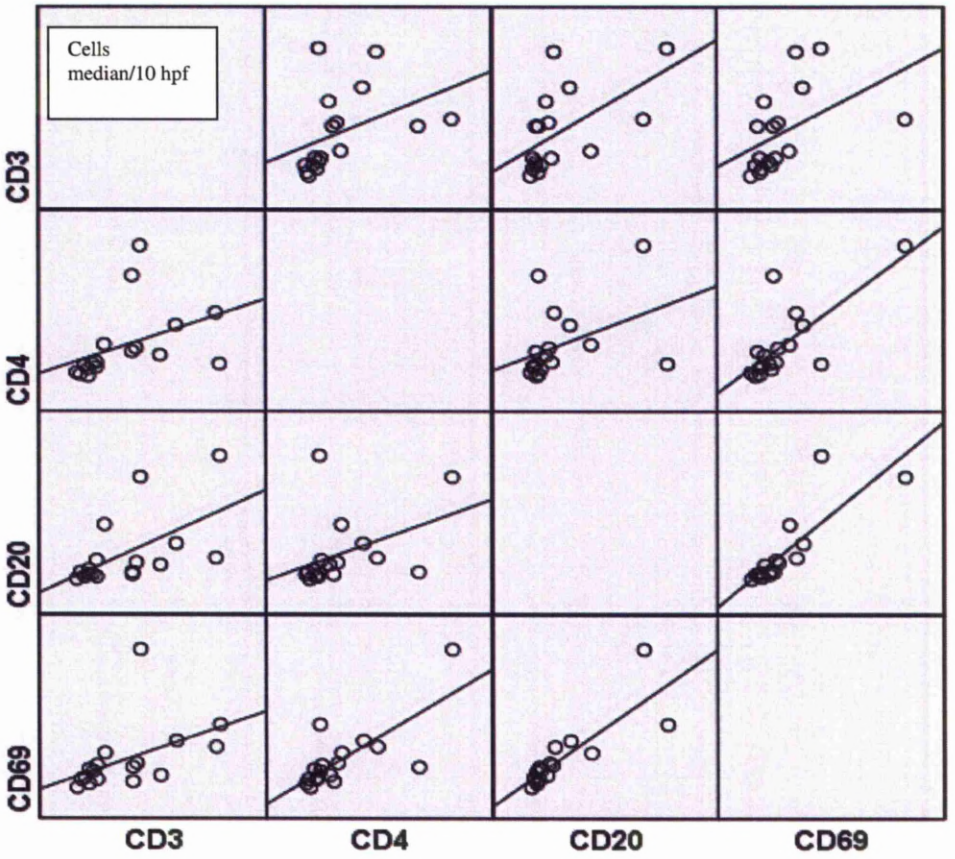


Figure 3.9: Correlation matrix depicting the correlation between T and B cells (expressed as median per 10 hpf) with CD 69 (CD3 and CD69 r {correlation coefficient} = 0.74, CD3 and CD20 r = 0.74, CD3 and CD4 r = 0.75, p value<0.01).

3.1.5 Discussion

In this study granulocytes were found to be the predominant leucocyte in both the ASA group and the control group. In direct contrast to previous studies, this study also showed CD4 as the predominant T lymphocyte in both the ASA groups and CD16 is the predominant cell in the control group and there was no significant difference in the levels of the CD8 lymphocytes between the ASA+ve or ASA-ve groups.

Furthermore, what was interesting to note was that the ratio of the CD4 / CD8 (Table 3.2) was significantly lower in the ASA+ve group (1.37) when compared to the ASA-ve group (1.9) or the control group (2). When culture negative individuals were only analysed the ratio of the CD4 / CD8 (Table 3.3) was significantly lower in the ASA+ve group (1.1) when compared to the ASA-ve group (2) or the control group (2). This might suggest that the CD8 lymphocytes actually have a more cytotoxic role and hence the reduction in the ratio of helper to cytotoxic cells.

This might be proven if the sperm count in the ASA+ve group is also lowered. This has also been looked at, which showed the sperm count in the ASA+ve group to be much lower at 26 (11-40) when compared to the control group at 104 (73-119) x 10^6/ml, median and range respectively. This was highly statistically significant. The sperm count in the ASA–ve group was also higher in comparison to the ASA+ve group although it did not reach statistical significance.

An increase in the phagocytic activity of the leucocytes in men with antisperm antibodies may be expected as many of the spermatozoa would be opsonized by antibody. However, in this study, the median and the range of the CD45 counts in the ASA+ve group was higher at 11.1 (4.7-20.1) x 10^6/ml. This was not a statistically significant difference when compared to the ASA-ve group at 5.35 (3.15-25.15) x 10^6/ml.

Unfortunately, in this study there were patients with genital infections in the ASA group. This may, to a certain extent, explain the presence of an increased number of leucocytes in the ASA group, particularly the ASA+ve group. When patients with genital infections were excluded in our study and a subgroup analysis was performed, the CD45 count was lower in the ASA+ve group when compared to the ASA–ve group and thus establishes that there was no relationship between the presence of ASA and the number of leucocytes in the ejaculate.

These results mirror those of another study and suggest that, despite the fact that both abnormalities are manifestations of an immunological response, they are not interrelated[48]. Leucocytopsermia was not significantly related to the presence of local antisperm antibodies of the IgG or IgA class. There was also no significant association of antisperm antibodies with the concentration of PMN elastase in seminal plasma and the outcome of semen cultures[198].

In this study, leucocytospermic samples with antisperm antibodies were associated with low sperm count, decreased motility, abnormal sperm morphology and poor SMTs. This association was highly significant (p=0.0001). It is also interesting to note that even in the ASA–ve group, leucocytospermia was associated with low sperm count, decreased motility,

abnormal sperm morphology and poor SMT. This association also reached statistical significance (p=0.002, 0.0001, 0.003 and 0.001 respectively).

This would lead one to believe that leucocytospermia, despite the presence or absence of ASA, could act as an individual variable in affecting sperm parameters directly[48]. However, leucocytes in ejaculates with negative bacterial cultures was not a representative parameter of fertilising ability as a variable number of leucocytes were found in the semen samples, and that leucocytes are a normal cellular component of human semen samples.

Maybe leucocytospermia alters semen quality only when seminal vesicles are also affected as the seminal vesicles produce antigens that could protect the sperm from immune response. Gonzales et al. also suggested that in conditions whereby function of the seminal vesicles becomes altered, the protecting antigen secreted by the seminal vesicles will be reduced; therefore, sperm antigens will sensitise leucocytes, resulting in an immune response with the production of antisperm antibodies[199].

Interferon produced by TH1 cells at high concentrations was noted to adversely affect sperm motility[200] and men with antisperm antibodies have lower sperm motility[201;202], suggesting the mechanism of the effect of antisperm antibodies on sperm motility may be via the action of IFN. This hypothesis needs to be specifically tested and this has been endeavoured to do so in the present study in Chapter 4 when discussing the role of cytokines in male infertility.

Returning to the hypothesis, there were no statistically significant differences observed in the leucocyte subpopulations between the ASA+ve and the ASA-ve groups in the current study.

3.2 STUDY NUMBER 2: Leucocyte subsets in patients with Positive cultures versus patients with Negative cultures

3.2.1 Introduction

Microorganisms present in the male genital tract and / or the ejaculate may often be difficult to detect in clinical andrology and pathology laboratories. The proteolytic and growth inhibitory properties of seminal fluid interfere with microbial growth *in vitro* and / or compromise the viability of cultured mammalian cells necessary for the replication of obligate intracellular microorganisms such as *Chlamydia trachomatis* or viruses. Microorganisms present in concentrations below the current limit of detection may, nevertheless, negatively influence sperm function. Microorganisms may bind to spermatozoa and compromise their ability to reach the fallopian tubes as well as their capacity for fertilisation.

Direct culture of semen is ideally the best practice method to detect male genital tract infection as it is 100% specific. However, these are technically demanding, needing cold chain particularly for *Chlamydia* to preserve specimen viability in transport and are not readily available. Furthermore, the results can take up to eight days.

Nucleic Acid Amplification Tests (NAAT) are both highly sensitive and specific (95%). The results of NAAT are received within a few hours. Thus, NAAT is replacing culture as the tests of choice in detecting male genital tract infections. However, to date there are no commercial NAAT tests available for testing semen[203].

The hypothesis was that leucocytospermia was present in the culture positive patients. The leucocyte subsets that are likely to be increased were the granulocyte and macrophages in the culture positive group. The aim of study 2 was to identify the presence of leucocytospermia and also analyse the activity of the different leucocyte subpopulations in the culture positive patients.

3.2.2 Materials and Methods

Direct culture of semen was used to identify male genital tract infections as outlined in Chapter 2 section 2.2.

3.2.3 Statistical Analysis

As outlined in Chapter 2 section 2.5.5.

3.2.4 Results

Out of the remaining 73 subfertile patients, 20 were found to be positive for *Ureaplasma* and 53 were negative. Of the 73 patients studied, 24 tested positive for general infections such as *Enterococcus* and *Streptococcus*. All the patients tested negative for *Mycoplasma, Chlamydia trachomatis* and *Trichomonas vaginalis*. There were no differences in the sperm parameters (numbers, motility, morphology) between the culture positive group and the culture negative groups (Tables 3.4 and 3.5). The levels of CD45 +ve cells and Granulocytes (CD16) were higher in the *Ureaplasma* positive group; however, these did not reach statistical significance (Table 3.6). Figures 3.10 and 3.11 represent the correlations that exist between T and B cells and granulocytes.

Table 3.4: Sperm parameters as median and ranges in Ureaplasma positive vs. Ureaplasma negative groups.

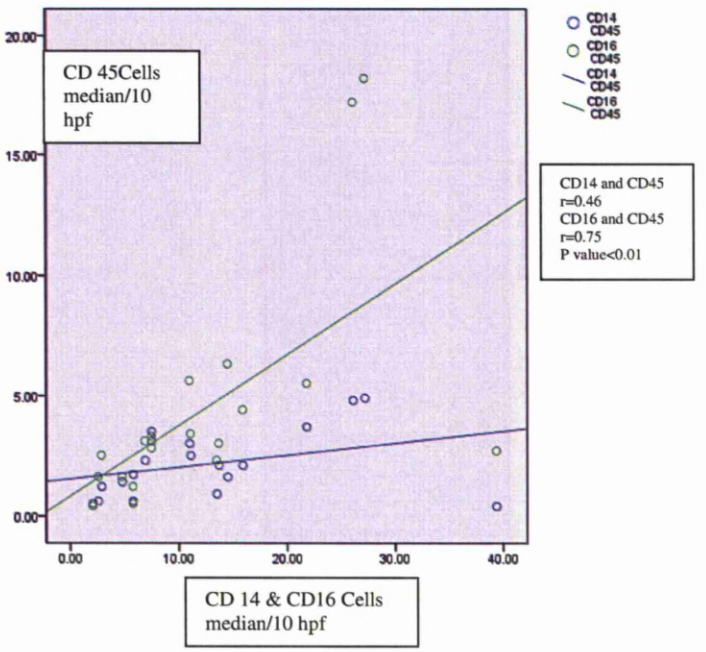
Sperm Parameters	Positive (n=20) Median and Ranges	Negative (n=53) Median and Ranges	P –value Positive vs. negative
Sperm count 10^6/ml	9 (0.8-18.5)	7 (0-29)	0.84
Motility %	7.5 (1-25)	10 (0-20)	0.83
Morphology %normal forms	11 (5-22)	10 (0-17)	0.56
SMT10^6/ml	0.3 (0-2)	0.1 (0-2.2)	0.86

Table 3.5: Sperm parameters in general culture positive vs. negative (parameters were unaffected in the presence of infection).

Sperm Parameters	Positive (n=24) Median and Ranges	Negative (n=46) Median and Ranges	P –value Positive vs. negative
Sperm count 10^6/ml	5 (0-28.5)	9 (0-34)	0.57
Motility %	15.5 (0-25)	10 (0-20)	0.46
Morphology %normal forms	11 (0-20)	10 (0-18)	0.74
SMT 10^6/ml	0.05 (0-2.75)	0.1 (0-1.5)	0.83

Table 3.6: Median and ranges of the cell surface markers (per 10 hpf) in Ureaplasma positive vs. Ureaplasma negative groups.

Antibodies	Positive (n=20) Median and Ranges	Negative (n=53) Median and Ranges	P – value Positive vs. negative
CD 45%	82(75-90)	77(63-89)	0.09
CD45	11 (5.8-15.9)	5.9 (3.7-11.8)	0.08
CD2	1.4 (0.6-2.6)	1.2 (0.6-1.9)	0.44
CD3	1.5 (0.8-2.9)	1.2 (0.6-1.9)	0.36
CD4	2 (1.1-3)	1.5 (0.8-2.5)	0.22
CD8	0.9 (0.7-1.1)	0.7 (0.3-1.7)	0.4
CD14	2.1 (0.9-3.1)	1.5 (0.7-3.5)	0.55
CD16	3 (1.6-5.5)	2.4 (1-5.4)	0.32
CD20	1 (0.6-2.2)	0.7 (0.3-2.3)	0.17
CD56	0.6 (0.2-1.4)	0.4 (0.3-0.8)	0.21
CD69	1.2 (0.7-2)	0.9 (0.3-1.7)	0.14
L 243	2.9 (2.3-4)	2.1 (1.3-4.9)	0.12



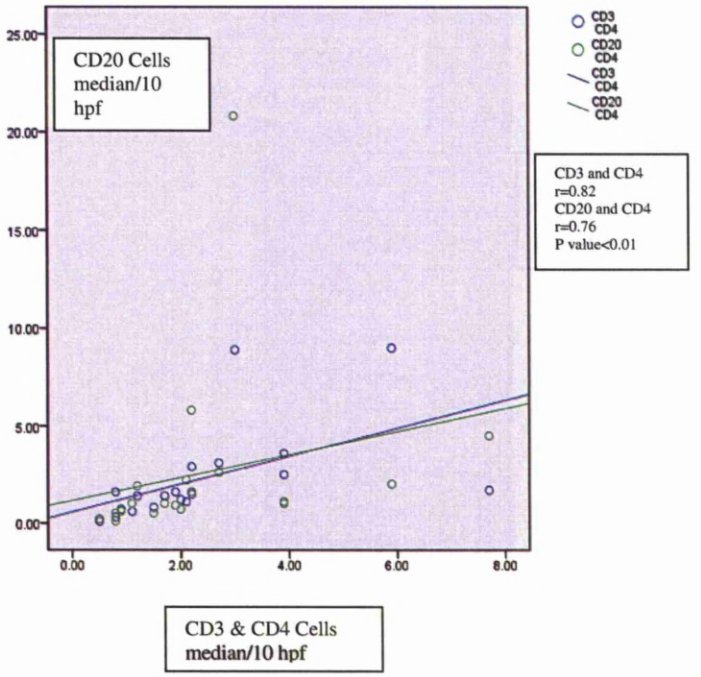


Figure 3.10: Correlation between macrophages and granulocytes (expressed as cells per 10 hpf) and between T and B cells in the Ureaplasma positive group.

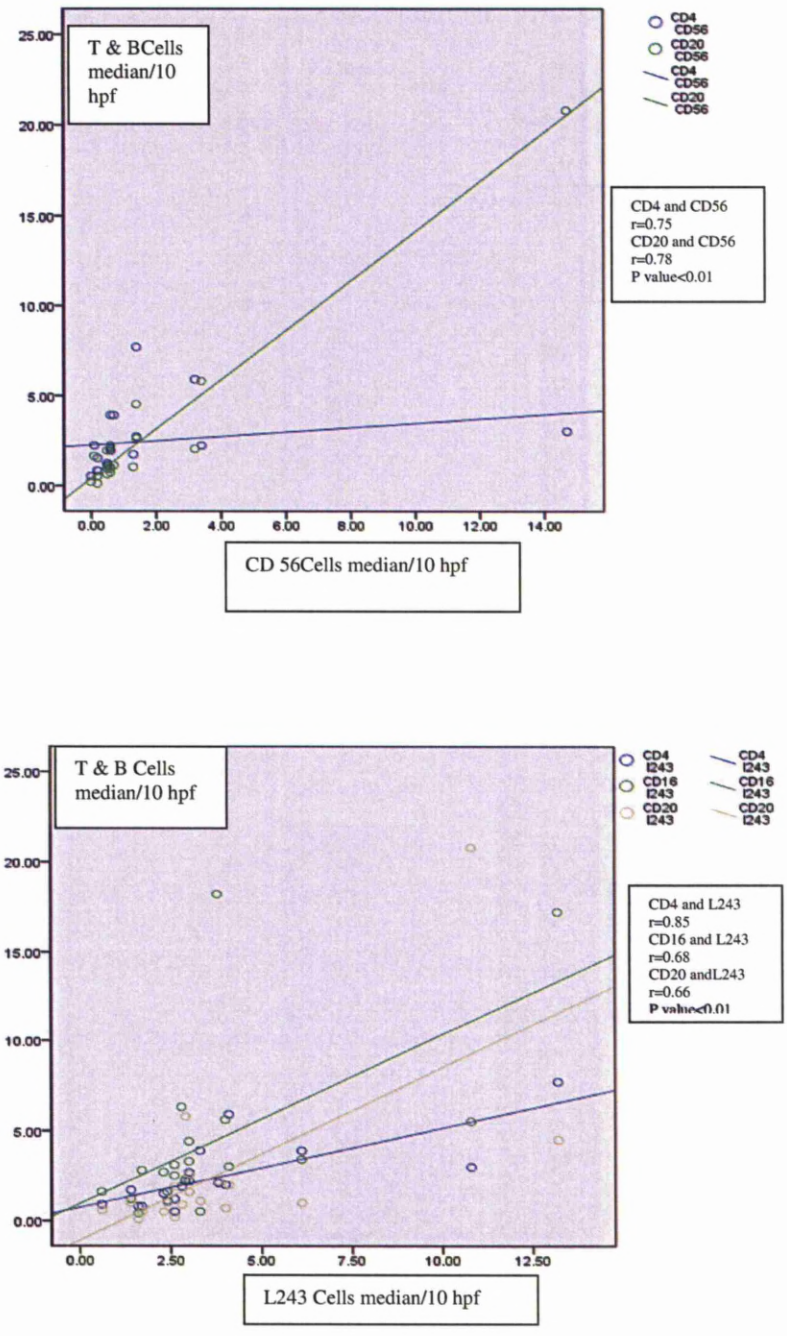


Figure 3.11: Correlation between T cells, B cells and large granular lymphocytes (expressed as cells per 10 hpf) in Ureaplasma positive group.

Table 3.7: Median and ranges of antibodies (expressed as cells per 10 hpf) in general culture positive vs. negative. (Culture positive included Enterococcus, Mixed Anaerobes, Streptococcus milleri and Beta haemolytic streptococcus.) * Significance at 5% level p value < 0.05, CD45, CD14 and CD16 levels were significantly higher in the culture negative group.

Antibodies	Positive (n=24) Median and Ranges	Negative (n=46) Median and Ranges	P–value Positive vs. negative
CD45%	75(63-87)	79(70-92)	0.06
CD45	4.65 (3.15-10.3)	7.2 (4-14.85)	**0.05***
CD2	1.25 (0.5-1.6)	1.45 (0.65-2.35)	0.13
CD3	1 (0.5-1.65)	1.55 (0.6-2.85)	0.14
CD4	1.4 (0.95-2.1)	1.75 (0.8-3.1)	0.32
CD8	0.75 (0.5-0.95)	0.9 (0.4-2)	0.36
CD14	1.35 (0.65-2)	2 (0.8-4.9)	**0.05***
CD16	1.9 (0.85-3.15)	3 (1.65-6.25)	**0.03***
CD20	0.7 (0.3-1.25)	1 (0.4-3.3)	0.1
CD56	0.4 (0.2-0.65)	0.5 (0.3-1.3)	0.09
CD69	0.7 (0.3-1.2)	1.4 (0.4-1.85)	0.11
L 243	2.45 (1.45-3.15)	2.35 (1.6-6.3)	0.36
CD4/CD8 Ratio	1.8	1.9	

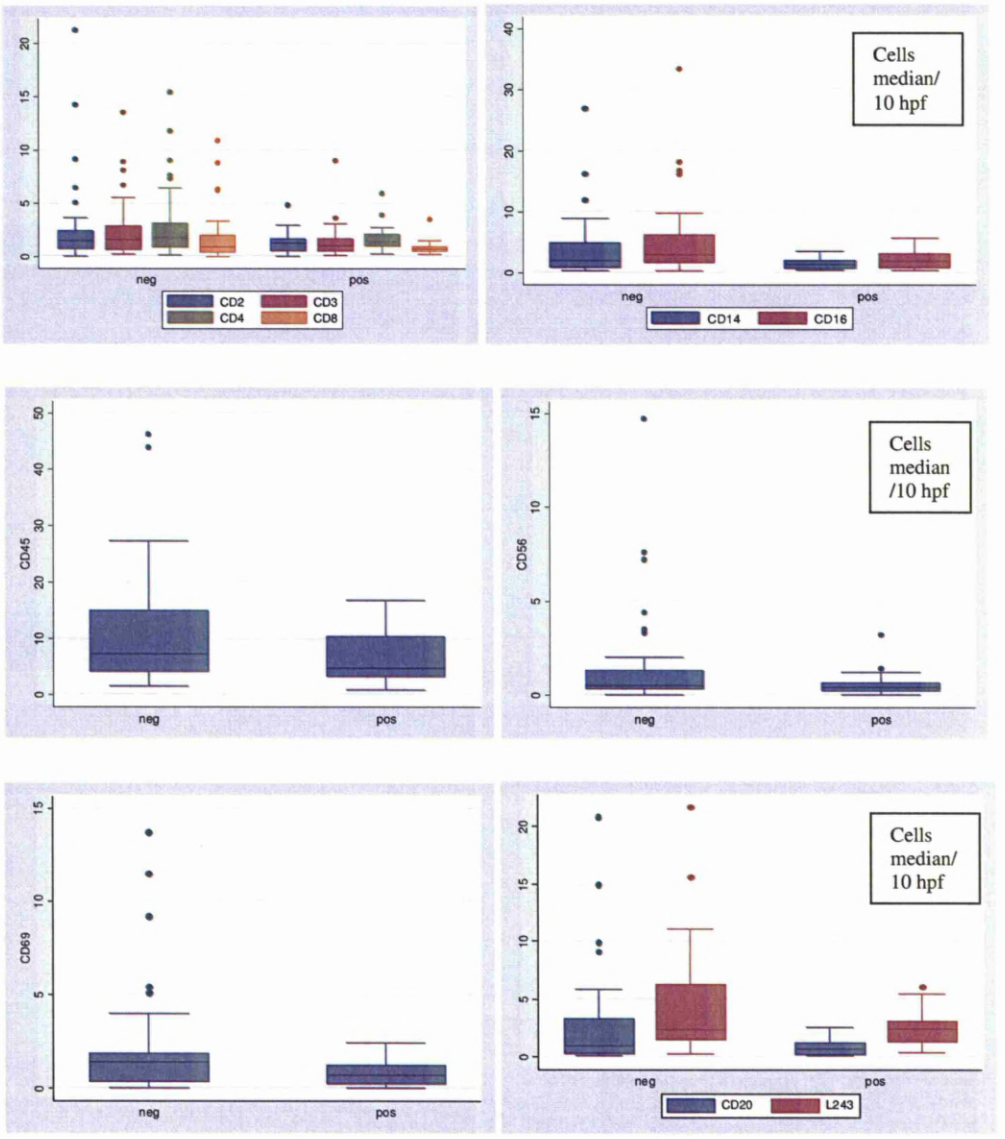
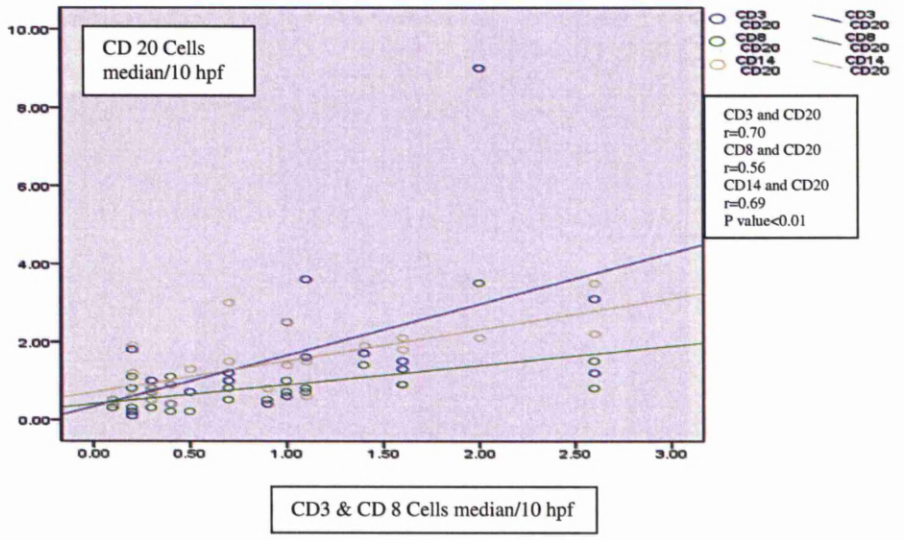


Figure 3.12: Box plots representing leucocytes (expressed as cells per 10 hpf) in general culture positive vs. negative. (Culture positive included Enterococcus, Mixed Anaerobes, Streptococcus milleri and Beta haemolytic streptococcus.) CD45, CD14 and CD16 are statistically significantly different between the two groups (p value<0.05).



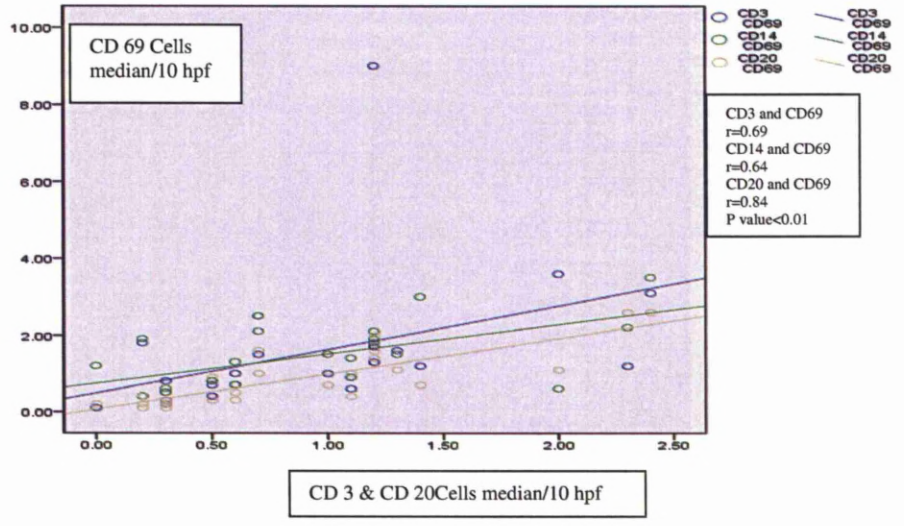


Figure 3.13: Correlation between leucocytes (expressed as cells per 10 hpf) in general culture positive group.

3.2.5 Discussion

This study failed to detect any statistical significant difference between the *Ureaplasma* positive and the *Ureaplasma* negative groups with regards to sperm parameters, particularly with reference to morphology or motility. It was also demonstrated that there was no statistical significant difference with regards to sperm count and SMT between the *Ureaplasma* positive and negative groups as shown in Table 3.4. Similarly, other study groups have also been unable to confirm any association between *Ureaplasma* infections and infertility or sperm quality[204].

Furthermore, there was no statistically significant leucocytopsermia amongst the *Ureaplasma* positive and *Ureaplasma* negative groups as shown in Table 3.6. The findings from this study would suggest that *Ureaplasma* is unable to mount a severe inflammatory response and *Ureaplasma urealyticum*, which is so widespread in the normal reproductive tract, can increase in numbers simply because the local immune system is attenuated by a primary infection and hence, its aetiological significance is weakened.

However, *Ureaplasma urealyticum* has been considered an aetiological agent in male infertility ever since the early observation that these organisms can attach firmly to spermatozoa[205]. Indeed, when *Ureaplasma urealyticum* has been implicated as a causative factor, it has often been associated with reduced sperm motility and poor sperm morphology[206]. *Mycoplasma* infections also appear to be associated with an increase in the percentage of coiled sperm tails[207] and infected sperm exhibit reduced oocyte penetration[208]. *Mycoplasma genitalium* and *Ureaplasma urealyticum* have strong adhesive properties and can bind to the sperm head and midpiece and negatively influence motility[209].

The antibody- and complement-coated spermatozoa would also be susceptible to phagocytosis by neutrophils and macrophages which possess complement or immunoglobulin Fc receptors. In many cases in which tetracycline therapy was initiated, varying grades of improvement in sperm quality have been recorded. The incidence of *Ureaplasma* infection has been reported to be higher amongst couples with idiopathic infertility.

Unfortunately, it is impossible to comment on the role of *Chlamydia* and sperm parameters and leucocyte count in the seminal plasma as none of the patients in this study tested positive for *Chlamydia*. It could be argued that one of the potential weaknesses of this study was the non utilisation of molecular laboratory techniques to identify Chlamydial infection. However, to overcome similar issues, a study recruiting men from a primary care setting found semen of men who tested positive for Chlamydial DNA were found to have significantly increased ejaculate volumes, leucocyte numbers and did not contain increased numbers of dead sperm [210]. This finding was supported by another study which found that sperm quality was unaffected by past or current Chlamydial infection[211]. However, this study went on to show that conception was negatively affected by previos Chlamydial infections. This was thought to be due to the affliction of the seminal vesicles by these organisms and thus decrease the antioxidant production which could leave the sperm more susceptible for free radical damage particularly during assisted reproduction techniques. Chlamydial infection is thought to affect sperm DNA fragmentation particularly when coinfected with *Mycoplasma*[212].

The role of *Chlamydia trachomatis* has been documented to be one of the other main aetiological agents in urethritis and acute epididymitis. The route of spread of the organism is intracanalicular, possibly secondary to urinary reflux into the vasa caused by the structural

changes in the ejaculatory ducts. It also implies that non-symptomatic *Chlamydia prostatitis* may precede acute epididymal infection[213]. Negative cultures of *Chlamydia trachomatis* from prostatic fluid or semen should not be taken as definitive proof that the bacteria is not present, because of its intracellular nature and the fact that male reproductive fluids can exert an inhibitory influence on the culture cells used to demonstrate the organism[214]. Up to 50% of men infected with *Chlamydia* remain asymptomatic[215].

A number of studies show that no relationship could be established between sperm quality and a Chlamydial infection, previous or present using culture or serology. Several factors could be considered in this respect; *Chlamydia trachomatis* constitutes a weak antigenic stimulus to the immune system, resulting in only modest antibody responses. An immunological response cannot be detected in approximately 20% of infected males. Seminal IgA could reduce the false positive tests that could result from cross reaction with non genital species when serum IgG is measured[216]. *Chlamydia* is associated with deep seated infection and a urethral discharge may not be appropriate in the majority of cases.

Chlamydia trachomatis may influence infertility by inducing sperm antibodies[28]. In men with Mycoplasmal or Chlamydial infections there was a highly significant increase (14 vs. 1.2%) in the number with sperm antibodies in their seminal plasma compared to non-infected controls[217]. Infection may also impair more subtle aspects of sperm quality not registered by conventional techniques. It may be significant in this respect that a special form of jerking movement has been registered by automatic motility analysis of sperm from men infected with *Chlamydia trachomatis* which is normalised after treatment with tetracycline[218].

Co-incubation with Chalmydial serovar was found to cause a statistically significant decline in the percentage of motile sperm and a dose dependant caspases mediated increase in the proportion of dead spermatozoa[219]. This was attributed to *Chlamydia's* potential to release lipopolysaccharide (LPS) which can be a 550 fold more potent spermicide when compared to Gram-ve LPS[220]. *Chlamydia* can also cause increased tyrosine phosphorylation of sperm proteins which is receptor mediated and thus affect sperm function as sperm capacitation is mediated through the phosphorylation process[219].

Furthermore, increased incidence of *Chlamydia trachomatis* has been found in men with obstructive azoospermia[221]. The mechanism responsible for this induction of antibodies is still unknown. Inflammation may lead to the migration of immune cells into the genital tract which then reacts to spermatozoa, which may be trapped in excurrent ducts. Alternatively, antibodies may be formed to common antigens between the bacteria and spermatozoa.

Due to the lack of positive patients in this study, it is difficult to either support or refute the role played by *Chlamydia trachomatis* infection in male subfertility. However, the issues have been discussed with regards to *Chlamydia* and male subfertility. Some of the seminal cultures tested positive for *Enterococcus*, mixed anaerobes, *Streptococcus milleri* and beta haemolytic *Streptococcus*. The sperm parameters were unaffected due to the presence of these infections as shown in Table 3.6.

No correlation was found between sperm characteristics and positive bacterial cultures and in semen in this study. The findings of this study mirror the findings of another study[101]. This would suggest that subclinical genital tract infection in asymptomatic patients has no major role in male infertility. There was no significant difference in the CD4 / CD8 ration between

the general culture positive and the culture negative group. Surprisingly, the CD45, CD14 and CD16 levels were significantly higher in the culture negative group as shown in Table 3.7. Thus it could be inferred that an increase in the macrophages in the culture negative group may be due to the need for phagocytosis of post capacitated human spermatozoa[222]. The correlations between the T and B cells have been shown again in Figure 3.15. This shows that activation of T cells leads to an activation of B cells thus providing the link in cell mediated immunity.

Although leucocytospermia is commonly found in genital tract infections, high concentrations of seminal WBC also occur in infertility patients in whom microbiological investigations, including *Chlamydia* and *Mycoplasma*, have revealed no bacterial agents. Such patients may be asymptomatic or may have clinical symptoms and / or ultrasonographic signs of an inflammation e.g. prostatovesiculitis[223]. Patients with clinical symptoms of an inflammatory process, despite negative bacterial cultures, might have an encapsulated infection or an infection caused by a virus.

A genital tract infection might cause release of toxic products from the microorganisms or changes in the biochemistry of seminal plasma. E.g. concentration of fructose, in itself affecting sperm function[199]. Genital tract infections have been associated with the formation of autoantibodies to the sperm in man[90]. It has been proposed that activation of T lymphocytes by a *Chlamydia trachomatis* infection initiates the development of an autoimmune response to spermatozoa in the male genital tract[224]. There have also been a large number of studies on the effects of antibiotic therapy on sperm quality and pregnancy rates, many of which have been contradictory.

There may be several reasons for difficulties in showing an antibiotic effect on male infertility: 1) the therapy may not have been appropriate for the organism responsible or it may not have been of adequate dose or duration; or 2) the pathological changes in the reproductive tract responsible for the poor sperm quality may be permanent.

In conclusion, analysing the leucocyte responses to bacterial infections, it was found that there was no statistically significant increase in the leucocyte count in the *Ureaplasma* positive group. This would suggest that this organism is incapable of mounting a severe inflammatory response. Also, other subclinical bacterial infections are unable to mount a sufficient inflammatory response. However, contrary to the hypothesis, in the general culture negative group the CD45, CD14 and CD16 numbers were increased. This would suggest that the increase in these cells is for removal of post capacitated spermatozoa.

3.3 STUDY NUMBER 3: Leucocyte subsets in Oligospermic versus the Normospermic group of patients

3.3.1 Introduction

Increased PMN concentrations are related to decreased percentages of normal forms of spermatozoa and a significant increase in the percentage of midpiece abnormalities[225]. At two different cut off points of seminal leucocytic concentration ($0.5x10^6$ and $1x10^6$ leucocytes per ml), the patient group with lower mean leucocyte concentration had a significantly higher proportion of sperm with normal morphology compared with patients who had a higher mean leucocyte concentration. There was also a significant positive correlation between leucocyte concentration and midpiece defects. No other correlation was detected[225].

A rise in the WBC concentration in semen was found due to an increased occurrence of morphologically abnormal sperm[226]. Another study has suggested a reduction of morphologically abnormal sperm by phagocytic activity in men with higher concentrations of WBC[202]. It is surprising that ejaculates from normal fertile men often continuously contain considerable numbers of morphologically abnormal sperm but only a few WBC. The peroxidase test determined WBC count had no statistically significant effect on sperm morphology[227].

The aim of the third study was to analyse the effect of seminal leucocyte subpopulations on various sperm parameters (count, motility, morphology and migration test) with particular emphasis on the sperm count. The hypothesis was that elevated numbers CD14 and CD16 positive cells macrophages and granulocytes would be associated with the oligospermic group. Presence of these cells being associated with abnormal sperm structure.

3.3.2 Materials and Methods

Immunohistological staining using monoclonal antibodies was carried out as outlined in Chapter 2 sections 2.5.1, 2.5.2 and 2.5.3.

3.3.3 Statistical Analysis

The analysis was performed as described in Chapter 2 section 2.5.5.

3.3.4 Results

13 (Mild-6) (Severe-7) oligospermic men were identified and compared to 14 normospermic men. The age of men ranged from 22-49 years (median 34.5 years). The duration of infertility was between 2-13 years (median 5 years).

Thirty eight of the men consumed alcohol on an average of 10-12 units/week. Twenty of the men also smoked on average 10-20 cigarettes per day.

Mild oligospermia was defined a sperm count of between 10-20x10^6 and a motility of >40% and severe oligospermia was defined as a sperm count of <10x10^6 and motlity of >40%. As expected, the sperm count in the mild (14.5 x 10^6/ml) and severe (5 x 10^6/ml) oligospermic group was significantly reduced when compared to the normospermic group (43 x 10^6/ml). The SMT and sperm morphology was significantly reduced in the severe oligospermic group as outlined in Table 3.8. The CD45 count was increased in the mild and severe oligospermic group compared to the normospermic group but did not reach statistical significance as seen in Table 3.9. The T lymphocytes (CD2, CD3, CD4 and CD8) were found predominantly in the mild and severe oligospermic group compared to the normospermic group. Only CD3 was significant. Granulocytes also formed a major percentage (29%) of the leucocytes in the oligospermic group. The MHC class 2 cells (L243) were found in all three groups.

The sperm parameters and cell surface receptor expression of the oligospermic group were compared to the control group and the results are outlined in Table 3.10. All the values were statistically significant. Figure 3.14 also shows the difference in the distribution of T and B cells in the three groups. CD3 was significantly elevated in the oligospermic group when compared to the normospermic group which could suggest an increased T cell helper activity in the oligospermic group. However, when compared to the control group all the cell surface receptor levels were significantly elevated in both the oligospermic and the normospermic group.

Table 3.8: Median and ranges of the sperm parameters in the oligospermic group vs. the normospermic group. ** Significance at the 1% level *significance at 5% level.

Sperm Parameters	Mild Oligospermic group (n=6)	Severe Oligospermic group (n=7)	Normospermic group (n=14)	Mild Oligospermic group vs. Normospermic group P value	Severe Oligospermic group vs. Normospermic group P value
Sperm count 10^6/ml	14.5 (10-19)	5 (5-9)	43 (28-75)	**0.0001****	**0.0001****
Motility %	28.75 (27.5-30)	27.5 (25-30)	25 (20-40)	0.84	0.64
Morphology % normal forms	22 (17-26)	18 (10-21)	28 (18-32)	0.15	**0.01****
SMT 10^6/ml	2 (1-4)	0.5 (0.1-0.5)	8 (2.5-12)	**0.03***	**0.001****

Table 3.9: Median and ranges of the leucocytes (expressed as cells per 10 hpf) in the mild and severe oligospermic group vs. the normospermic group. *Significance at 5% level, **significance at the 1% level

	Mild Oligospermic (n=6) Median and ranges	Severe Oligospermic (n=7) Median and ranges	Normospermic (n=14) Median and ranges	P value Normospermic vs. Mild Oligospermic	P value Normospermic vs. Severe Oligospermic	P value Normospermic vs. Oligospermic
CD 45%	79(67-96)	75(27-89)	63(55.5-76)	0.14	0.41	0.43
CD 45	7.65 (4-26.1)	10.3 (4.5-13.5)	5.9 (3.9-16.7)	0.82	0.7	0.69
CD 2	1.45 (0.6-2.2)	1.6 (1.3-2.4)	0.7 (0.3-1.9)	0.48	0.2	0.21
CD 3	1.25 (0.6-2.2)	1.8 (1.6-2.9)	0.5 (0.3-1.3)	0.11	**0.002****	**0.003****
CD 4	1.5 (0.8-3.9)	1.8 (1.1-2)	1 (0.6-2.2)	0.46	0.44	0.34
CD 8	0.75 (0.7-1.9)	0.7 (0.3-0.9)	0.6 (0.2-1.1)	0.24	0.8	0.39
CD 14	0.65 (0.4-2.3)	1.6 (0.9-1.9)	1.2 (0.5-2.8)	0.56	0.44	0.87
CD 16	1.6 (0.5-2.7)	3 (2.3-5.7)	2.5 (1.5-5.7)	0.27	0.67	0.71
CD 20	0.65 (0.3-1.1)	0.9 (0.2-1.1)	0.4 (0.2-1)	0.58	0.55	0.47
CD 56	0.5 (0.2-0.9)	0.6 (0.2-0.9)	0.4 (0.3-0.5)	0.75	0.48	0.52
CD 69	0.5 (0.4-2)	1.3 (1.2-1.8)	0.5 (0.2-1.2)	0.7	0.09	0.18
L243	2.4 (1.1-3.3)	2.5 (1.7-2.9)	2.4 (1.7-5.5)	0.79	0.89	0.80

Table 3:10: Medians and ranges of the sperm parameters, CD counts (expressed as cells per 10 hpf) in the oligospermic group vs. the control group. ** Significance at the 1% level *significance at the 5% level.

	Mild Oligospermic group (n=6)	Severe Oligospermic group (n=7)	Controls (n=26) Median and ranges	Mild Oligospermic group vs. Controls P value	Severe Oligospermic group vs. Controls P value
Sperm Count 10^6/ml	14.5 (10-19)	5 (5-9)	104 (73-119)	0.0001**	0.0001**
Motility %	28.75 (27.5-30)	27.5 (25-30)	90 (80-99)	0.0001**	0.0001**
Morphology %normal forms	22 (17-26)	18 (10-21)	41 (31-48)	0.001**	0.0001**
SMT 10^6/ml	2 (1-4)	0.5 (0.1-0.5)	24 (15-30)	0.0001**	0.0001**
CD 45%	79(67-96)	75(27-89)	76(69-90)	0.83	0.48
CD 45	7.65 (4-26.1)	10.3 (4.5-13.5)	2.25 (0.65-4.7)	0.0001**	0.0001**
CD 2	1.45 (0.6-2.2)	1.6 (1.3-2.4)	0.15 (0-0.55)	0.0001**	0.0001**
CD 3	1.25 (0.6-2.2)	1.8 (1.6-2.9)	0.15 (0.05-0.65)	0.0001**	0.0001**
CD 4	1.5 (0.8-3.9)	1.8 (1.1-2)	0.2 (0-0.3)	0.0001**	0.0001**
CD 8	0.75 (0.7-1.9)	0.7 (0.3-0.9)	0.1 (0-0.4)	0.0001**	0.0001**
CD14	0.65 (0.4-2.3)	1.6 (0.9-1.9)	0.55 (0.1-2.25)	0.002**	0.0001**
CD16	1.6 (0.5-2.7)	3 (2.3-5.7)	0.65 (0.05-1.05)	0.0001**	0.0001**
CD20	0.65 (0.3-1.1)	0.9 (0.2-1.1)	0.2 (0-0.6)	0.0001**	0.0001**
CD56	0.5 (0.2-0.9)	0.6 (0.2-0.9)	0.1 (0-0.35)	0.011**	0.0001**
CD69	0.5 (0.4-2)	1.3 (1.2-1.8)	0 (0-0.1)	0.003**	0.0001**
L 243	2.4 (1.1-3.3)	2.5 (1.7-2.9)	0.7 (0.3-1.8)	0.0001**	0.0001**
CD4/CD8 Ratio	2	2.5	2		

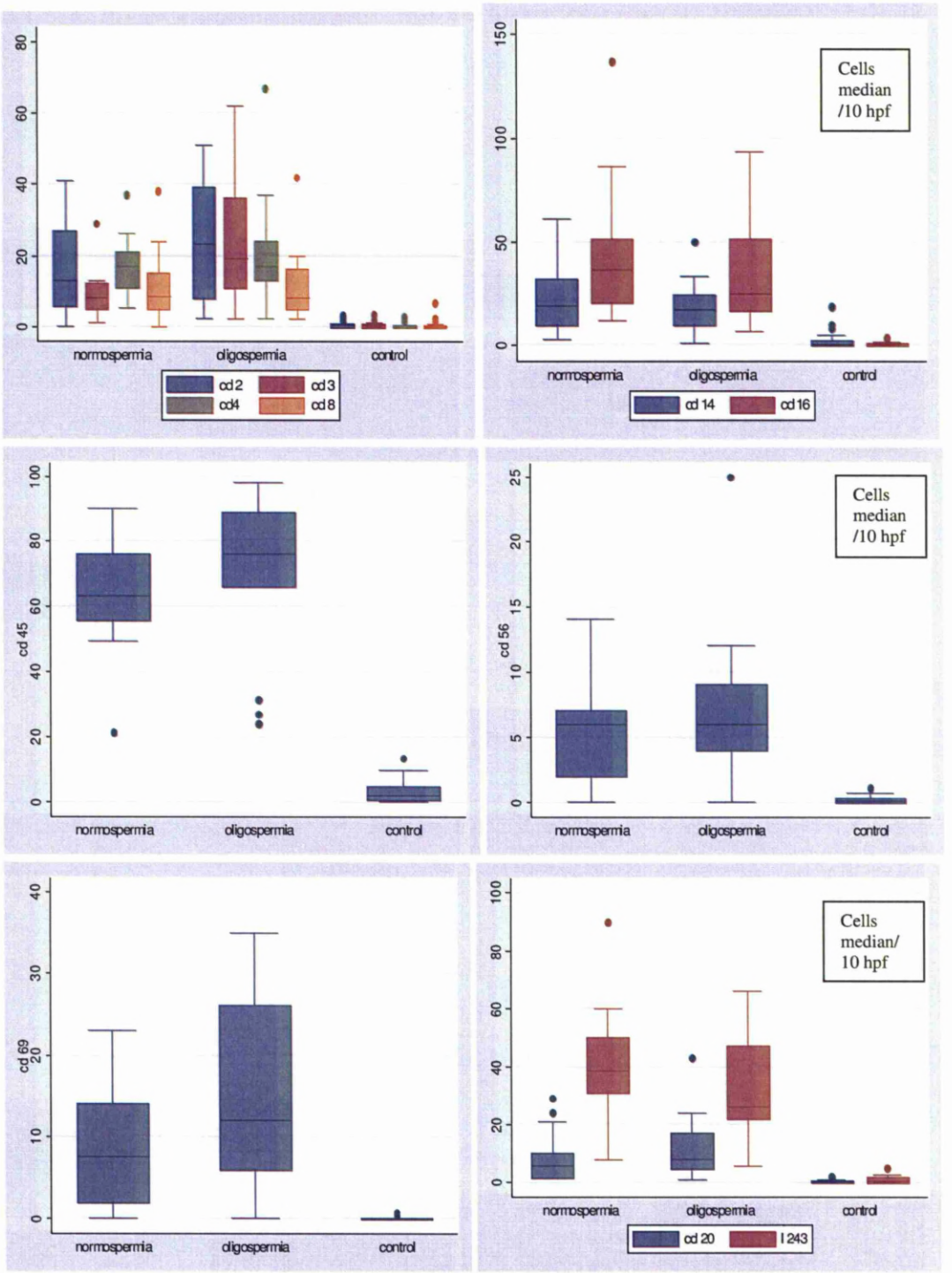


Figure 3.14: Box plots representing the leucocytes expressed as (cells per 10 hpf) in the oligospermic group, normospermic group and controls.

3.3.5 Discussion

Leucocytospermia was shown to impair sperm function through reduced antioxidant activity and enhanced T helper 1 modulation[228]. This study showed for the first time that significant levels of CD3 helper T lymphocytes were present in the oligospermic group compared to the normospermic group. Lymphocytes subpopulations have been demonstrated in the rete testis, epididymis, vas deferens, prostate and seminal vesicles[229].

The ratio of T helper cells to T cytotoxic cells is altered in the different subgroups of male infertile patients. This ratio was not altered between the mild oligospermic group and the control group which was 2 when compared to the severe oligospermic group which was 2.5. This would suggest an increase in the helper T cell activity in this group. There is no explanation for this finding.

There was no increase in the CD14 and CD16 cells in the oligospermic group when compared to the normospermic group contrary to the hypothesis. This would suggest that these leucocyte subpopulations are not a major factor in influencing sperm concentration.

3.4 STUDY NUMBER 4: Leucocyte subsets in Asthenospermic versus the Normospermic group of patients

3.4.1 Introduction

A potential mechanism through which leucocytospermia may induce alteration in sperm function is excessive ROS production by activated granulocytes. It has been argued that oxidative stress may induce alterations in the regulation of spermatogenesis, resulting in structural defects of the sperm[230;231].

On the subcellular level, it has been shown that leucocytospermia and excessive ROS levels are associated with an increase in chromatin alterations and DNA damage in sperm, as defined by the sperm chromatin structure assay[232].

On a different level, it has been shown that a high incidence of sperm tail defects is associated with sperm chromosomal abnormalities[233]. It is thus conceivable that peroxidative genetic damage may have led to a significant increase in the proportion of sperm with tail defects observed in the leucocytospermic study population.

The aim of the fourth study was to determine if there was a relationship between various seminal leucocyte subpopulations and sperm motility.

3.4.2 Materials and Methods

Immunohistological staining using monoclonal antibodies was carried out as outlined in Chapter 2 sections 2.5.1, 2.5.2 and 2.5.3.

3.4.3 Statistical Analysis

As outlined in Chapter 2 section 2.5.5.

3.4.4 Results

10 asthenospermic men were identified and compared to 14 normospermic men. Asthenospermia was defined as a sperm count $>20x10^6$ and a motility of <40%. On comparison with the normospermic group, the sperm motility, SMT and the sperm morphology were significantly reduced in the asthenospermic group as shown in Table 3.11.

The predominant leucocyte subgroup was the granulocytes in the normospermic and the asthenospermic group. The CD3 and the CD20 levels were significantly increased in the asthenospermic group (2.35 and 1.85 x 10^6/ml) when compared to the normospermic group (0.5 and 0.4 x 10^6/ml) respectively as shown in Table 3.12.

A further comparison between the asthenospermic group and the control group was performed. The results are outlined in Table 3.13. All the parameters were significantly increased in the asthenospermic group. Figures 3.15 and 3.16 depict the CD3 and CD20 cells stained pink which were significantly elevated in the asthenospermic group.

Table 3.11: Median and ranges of the sperm parameters in the asthenospermic group vs. the normospermic group. ** Significance at the 1% level.

Sperm Parameters	Normospermic group (n=14) Median and ranges	Asthenospermic group (n=10) Median and Ranges	Asthenospermic group vs. Normospermic group (p value)
Sperm count 10^6/ml	43 (28-75)	46.5 (34-83)	0.8
Motility %	25 (20-40)	13.75 (2.5-17.5)	**0.0001****
Progression	30 (25-60)	33.75 (8-37.5)	0.85
SMT 10^6/ml	8 (2.5-12)	0.3 (0-1.5)	**0.001****
Morphology % normal forms	28 (18-32)	10 (9-12)	**0.003****

Table 3.12: Median and ranges of the leucocytes (expressed as cells per 10 hpf) in the asthenospermic group vs. the normospermic group. **Significance at the 1% level *significance at the 5% level.

Antibodies	Normospermic group (n=14) Median and ranges	Asthenospermic group (n=10) Median and ranges	Asthenospermic vs. Normospermic group p value
CD 45%	63 (55.5-76)	77 (69-79)	0.14
CD 45	5.9 (3.9-16.7)	12.3 (9.6-18.4)	0.39
CD 2	0.7 (0.3-1.9)	1.6 (1-4)	0.15
CD 3	0.5 (0.3-1.3)	2.35 (0.9-5)	**0.01****
CD 4	1 (0.6-2.2)	2.15 (1-3)	0.19
CD 8	0.6 (0.2-1.1)	0.95 (0.9-3.2)	0.06
CD 14	1.2 (0.5-2.8)	3.25 (1.8-4.5)	0.08
CD 16	2.5 (1.5-5.7)	3.85 (1.6-5.5)	0.82
CD 20	0.4 (0.2-1)	1.85 (1-5.4)	**0.02***
CD 56	0.4 (0.3-0.5)	0.4 (0.1-0.8)	0.8
CD 69	0.5 (0.2-1.2)	1.75 (0.7-3.1)	0.07
L243	2.4 (1.7-5.5)	2.8 (1.7-6.4)	0.93

Table 3.13: Medians and ranges of the sperm parameters and CD counts (expressed as cells per 10 hpf) in the asthenospermic group vs. the control group. ** Significance at the 1% level *significance at the 5% level.

	Asthenospermic group (n=10)	Controls (n=26) Median and ranges	Asthenospermic group vs. Controls P value
Sperm Count 10^6/ml	46.5 (34-83)	104 (73-119)	**0.01****
Motility %	13.75 (2.5-17.5)	90 (80-99)	**0.0001****
Morphology % normal forms	10 (9-12)	41 (31-48)	**0.0001****
SMT 10^6/ml	0.3 (0-1.5)	24 (15-30)	**0.0001****
CD 45%	77(69-79)	76 (69-90)	0.53
CD 45	12.3 (9.6-18.4)	2.25 (0.65-4.7)	**0.0001****
CD 2	1.6 (1-4)	0.15 (0-0.55)	**0.0001****
CD 3	2.35 (0.9-5)	0.15 (0.05-0.65)	**0.0001****
CD 4	2.15 (1-3)	0.2 (0-0.3)	**0.0001****
CD 8	0.95 (0.9-3.2)	0.1 (0-0.4)	**0.0001****
CD14	3.25 (1.8-4.5)	0.55 (0.1-2.25)	**0.0001****
CD16	3.85 (1.6-5.5)	0.65 (0.05-1.05)	**0.0001****
CD20	1.85 (1-5.4)	0.2 (0-0.6)	**0.0001****
CD56	0.4 (0.1-0.8)	0.1 (0-0.35)	**0.0001****
CD69	1.75 (0.7-3.1)	0 (0-0.1)	**0.0001****
L 243	2.8 (1.7-6.4)	0.7 (0.3-1.8)	**0.0001****
CD4/CD8 Ratio	2.2	2	

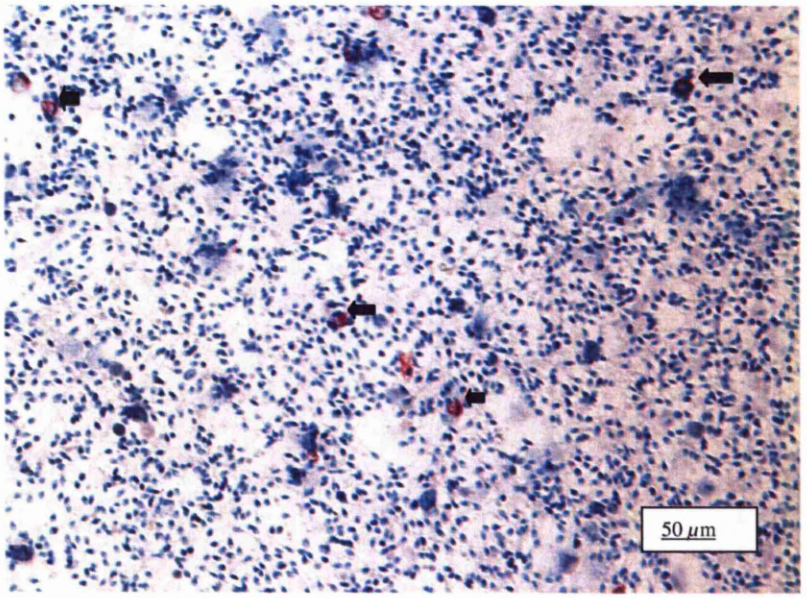


Figure 3.15: CD3 cells (stained pink in colour) in the asthenospermic sample.

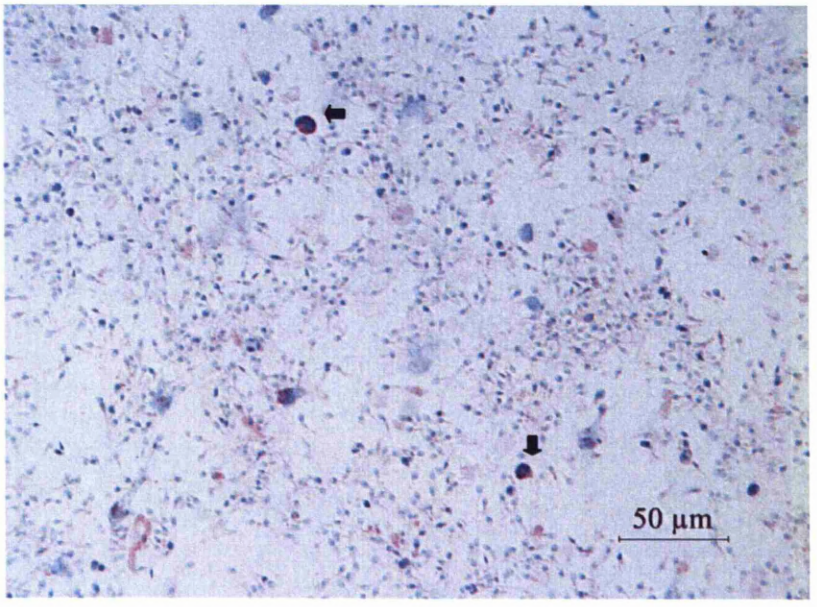


Figure 3.16: CD20 cells (stained pink) in the asthenospermic sample.

3.4.5 Discussion

This study showed for the first time that significant levels of CD3 helper T lymphocytes were present in the asthenospermic group when compared to the normospermic group. This would suggest an increase in the helper T cell activity in this group.

Significant levels of CD20 were also identified in the asthenospermic group when compared to the normospermic group. This would suggest that an increase in this cell group could affect sperm motility through an increased ASA production. This ASA could bind to the sperm and decrease its motility. The activation of a resting helper T cell causes it to release cytokines in particular IFN which is known to affect sperm motility. Similarly, activation of resting T helper cells could stimulate the activity of B cells (CD20), the latter producing antibodies and thus further decreasing sperm motility.

The correlation between leucocytospermia and male infertility is controversial: some studies have failed to find any association[226; 234]. However, there is evidence of sperm damage due to leucocytes: (i) seminal leucocytes numbers have been reported to be higher in infertile than in fertile male patients; (ii) leucocytospermia has been associated with decreased sperm concentration and motility; (iii) leucocytes damage sperm function and are an important prognostic factor for IVF-ET failure[102].

Other studies have demonstrated teratoasthenozoospermia and necrozoospermia in leucocytospermic men[100;225;227]. A significant correlation between leucocytospermia and sperm defects at acrosome and tail level was highlighted by another study[235]. Leucocytes are activated by different stimuli which generate an inflammatory response: the presence in the seminal fluid of inflammatory mediators might induce oxidative stress, alteration in sperm

motility, acrosome reaction and DNA integrity[111;114;232]. Different studies suggest a negative effect of leucocytes and reactive-oxygen species, primarily produced by leucocytes, on semen quality [236; 237].

This is the first study which reports raised T helper cells (CD3) and B cells (CD20) in the asthenospermic group.

3.5 STUDY NUMBER 5: Leucocyte subsets in Oligoasthenospermic versus the Normospermic group of patients

3.5.1 Introduction

Different hypotheses have been explored recently to explain the relationship between leucocytospermia and sperm structural damage. The hypotheses that sperm structural damage occurs during spermatogenesis is consistent with the findings of a study which showed that infertile men with and without leucocytospermia had similar sperm concentrations in semen[235]. Low sperm concentrations would have been expected if the leucocyte induced damage occurred during spermatocytogenesis (proliferative phase).

The mechanism through which leucocytospermia may induce the alteration in sperm structure is not clear. However, one potential explanation is that leucocytospermia could be a marker for an inflammatory process in the testis and in most cases would be related to a subclinical inflammatory process and not due to an overt epididymoorchitis. The presence of proinflammatory mediators such as cytokines in the testis could lead to alterations in the regulation of spermiogenesis by interfering with Sertoli cell function leading to abnormal spermiogenesis[238].

In addition, significantly higher proportions of sperm with cytoplasmic droplet in the leucocytospermic patient group could be due to defective Sertoli cell function and disorganised spermiation[235]. Alternatively, it could be due to alterations in the maturation process that the sperm undergo while transient in the epididymis.

Alterations in epididymal sperm maturation would also explain why leucocytospermic men have significantly lower sperm motility. Thus, leucocytospermia induced sperm damage may commence during spermiogenesis and continue through spermiation and epididymal migration.

Thus, leucocytospermia could induce sperm damage by a number of mechanisms such as: 1) DNA damage; 2) release ROS; 3) affect spermatogenesis; 4) lead to cytokine induced sperm damage; and 5) alteration in the sperm epididymal maturation which could lead to decreased sperm motility. The aim of the fifth study was to show the affect of various leucocyte subpopulations on sperm parameters with particular emphasis on sperm count and motility.

3.5.2 Materials and Methods

Immunohistological staining using monoclonal antibodies was carried out as outlined in Chapter 2 sections 2.5.1, 2.5.2 and 2.5.3.

3.5.3 Statistical Analysis

The analysis was performed as described in Chapter 2 section 2.5.5.

3.5.4 Results

19 (Mild-5, Severe- 14) oligoasthenospermic men were identified and compared to 14 normospermic men. Mild oligoasthenospermia was defined as a sperm count of between 10-20x10^6 and amotility of <40%. Severe oligoasthenospermia was defined as a sperm count of <10 x10^6 and motility of <30%.

All the sperm parameters were significantly reduced particularly the sperm count and motility in the mild (18 x 10^6/ml, 10%) and severe (3.6 x 10^6/ml, 5%) oligoasthenospermic group when compared to the normospermic group (43 x 10^6/ml, 25%) respectively (Table 3.14).

The T cells (CD 3 {1.3 x 10^6/ml} and CD8 {0.95 x 10^6/ml}), B cells (CD20 {1.15 x 10^6/ml}) and large granular lymphocytes (CD56 {0.7 x 10^6/ml}) were significantly raised in the severe oligoasthenospermic group compared to the normospermic group {0.5, 0.6, 0.4 and 0.4 x 10^6/ml} respectively (Table 3.15).

There seems to be a finely balanced interaction between the T cells and the B cells in the oligoasthenospermic group, increased T cell activity is more likely to be of a helper in nature, whereas the increased B cells activity might be the cause of decreased motility due to antibody production and the increased CD56 activity could be removing the non functioning sperm. The likely severity of the condition will probably depend of the predominance of the cell group.

Table 3.14: Median and ranges of the sperm parameters in the oligosthenospermic group vs. the normospermic group. ** Significance at the 1% level.

Sperm Parameters	Normospermic group (n=14) Median and ranges	Mild Oligoasthenospermic group (n=5) Median and ranges	Severe Oligoasthenospermic group (n=14) Median and ranges	Mild Oligoasthenospermic vs. normospermic P value	Severe Oligoasthenospermic vs. normospermic P value	Oligoasthenospermic vs. normospermic P value
Sperm count 10^6/ml	43 (28-75)	18 (15-18)	3.6 (1.6-7)	**0.001****	**0.0001****	**0.0001****
Motility %	25 (20-40)	10 (2.5-10)	5 (5-15)	**0.002****	**0.0001****	**0.0001****
SMT 10^6/ml	8 (2.5-12)	0.4 (0.1-1)	0 (0-0.4)	**0.004****	**0.0001****	**0.0001****
Morphology % normal forms	28 (18-32)	9 (9-12)	15 (8-16)	**0.003****	**0.0001****	**0.0001****

Table 3.15: Median and ranges of leucocytes (expressed as cells per 10 hpf) in mild and severe oligoasthenospermic group vs. the normospermic group. *Significance at 5% level **significance at the 1% level.

Antibodies	Normospermic group (n=14) Median and ranges	Mild Oligoasthenospermic group (n=5) Median and ranges	Severe Oligoasthenospermic group (n=14) Median and ranges	Mild Oligoasthenospermic vs. normospermic P value	Severe Oligoasthenospermic vs. normospermic P value	Oligoasthenospermic vs. normospermic P value
CD 45%	63(55.5-76)	77(72-77.3)	81.7(66-90)	0.38	0.07	0.09
CD 45	5.9 (3.9-16.7)	7.5 (4-8.2)	7.75 (4.1-12.4)	0.83	0.98	0.945
CD 2	0.7 (0.3-1.9)	1.9 (1.6-2)	1.85 (0.6-2.9)	0.11	0.19	0.09
CD 3	0.5 (0.3-1.3)	1.9 (1.7-2.4)	1.3 (0.5-6.7)	**0.003****	**0.04***	**0.004****
CD 4	1 (0.6-2.2)	2.4 (2-2.5)	1.7 (0.8-3.2)	0.18	0.35	0.2
CD 8	0.6 (0.2-1.1)	1.1 (0.9-1.7)	0.95 (0.7-2.6)	0.1	**0.04***	**0.02***
CD 14	1.2 (0.5-2.8)	2.8 (2-3.1)	1.9 (0.8-4.7)	0.29	0.31	0.22
CD 16	2.5 (1.5-5.7)	2.8 (2.5-8)	2.05 (1.2-4.7)	0.46	0.65	0.94
CD 20	0.4 (0.2-1)	0.5 (0.4-3)	1.15 (0.6-2.2)	0.16	**0.03***	**0.02***
CD 56	0.4 (0.3-0.5)	0.3 (0.3-1.1)	0.7 (0.5-1.9)	0.79	**0.02***	**0.05***
CD 69	0.5 (0.2-1.2)	1.7 (0.9-2.5)	1.05 (0.5-1.5)	0.17	0.16	0.1
L243	2.4 (1.7-5.5)	2.2 (1.7-4.9)	2.5 (1.6-4.1)	0.76	0.62	0.6

Table 3.16: Medians and ranges of the sperm parameters and leucocytes (expressed as cells per 10 hpf) in the oligosthenospermic group vs. the control group.

	Mild Oligoasthenospermic group (n=5) Median and ranges	Severe Oligoasthenospermic group (n=14) Median and ranges	Controls (n=26) Median and ranges	Mild oligoasthenospermic group vs. Controls P value	Severe Oligoasthenospermic vs. controls P value
Sperm Count 10^6/ml	18 (15-18)	3.6 (1.6-7)	104 (73-119)	**0.001****	**0.0001****
Motility %	10 (2.5-10)	5 (5-15)	90 (80-99)	**0.0001****	**0.0001****
Morphology %normal forms	9 (9-12)	15 (8-16)	41 (31-48)	**0.001****	**0.0001****
SMT 10^6/ml	0.4 (0.1-1)	0 (0-0.4)	24 (15-30)	**0.001****	**0.0001****
CD 45%	77(72-77.3)	81.7(66-90)	76(69-90)	0.66	0.89
CD 45	7.5 (4-8.2)	7.75 (4.1-12.4)	2.25 (0.65-4.7)	**0.0001****	**0.0001****
CD 2	1.9 (1.6-2)	1.85 (0.6-2.9)	0.15 (0-0.55)	**0.0001****	**0.0001****
CD3	1.9 (1.7-2.4)	1.3 (0.5-6.7)	0.15 (0.05-0.65)	**0.0001****	**0.0001****
CD4	2.4 (2-2.5)	1.7 (0.8-3.2)	0.2 (0-0.3)	**0.0001****	**0.0001****
CD8	1.1 (0.9-1.7)	0.95 (0.7-2.6)	0.1 (0-0.4)	**0.0001****	**0.0001****
CD14	2.8 (2-3.1)	1.9 (0.8-4.7)	0.55 (0.1-2.25)	**0.0001****	**0.0001****
CD16	2.8 (2.5-8)	2.05 (1.2-4.7)	0.65 (0.05-1.05)	**0.0001****	**0.0001****
CD20	0.5 (0.4-3)	1.15 (0.6-2.2)	0.2 (0-0.6)	**0.0001****	**0.0001****
CD56	0.3 (0.3-1.1)	0.7 (0.5-1.9)	0.1 (0-0.35)	**0.0001****	**0.0001****
CD69	1.7 (0.9-2.5)	1.05 (0.5-1.5)	0 (0-0.1)	**0.0001****	**0.0001****
L 243	2.2 (1.7-4.9)	2.5 (1.6-4.1)	0.7 (0.3-1.8)	**0.0001****	**0.0001****
CD4/CD8 Ratio	2.18	1.78	2		

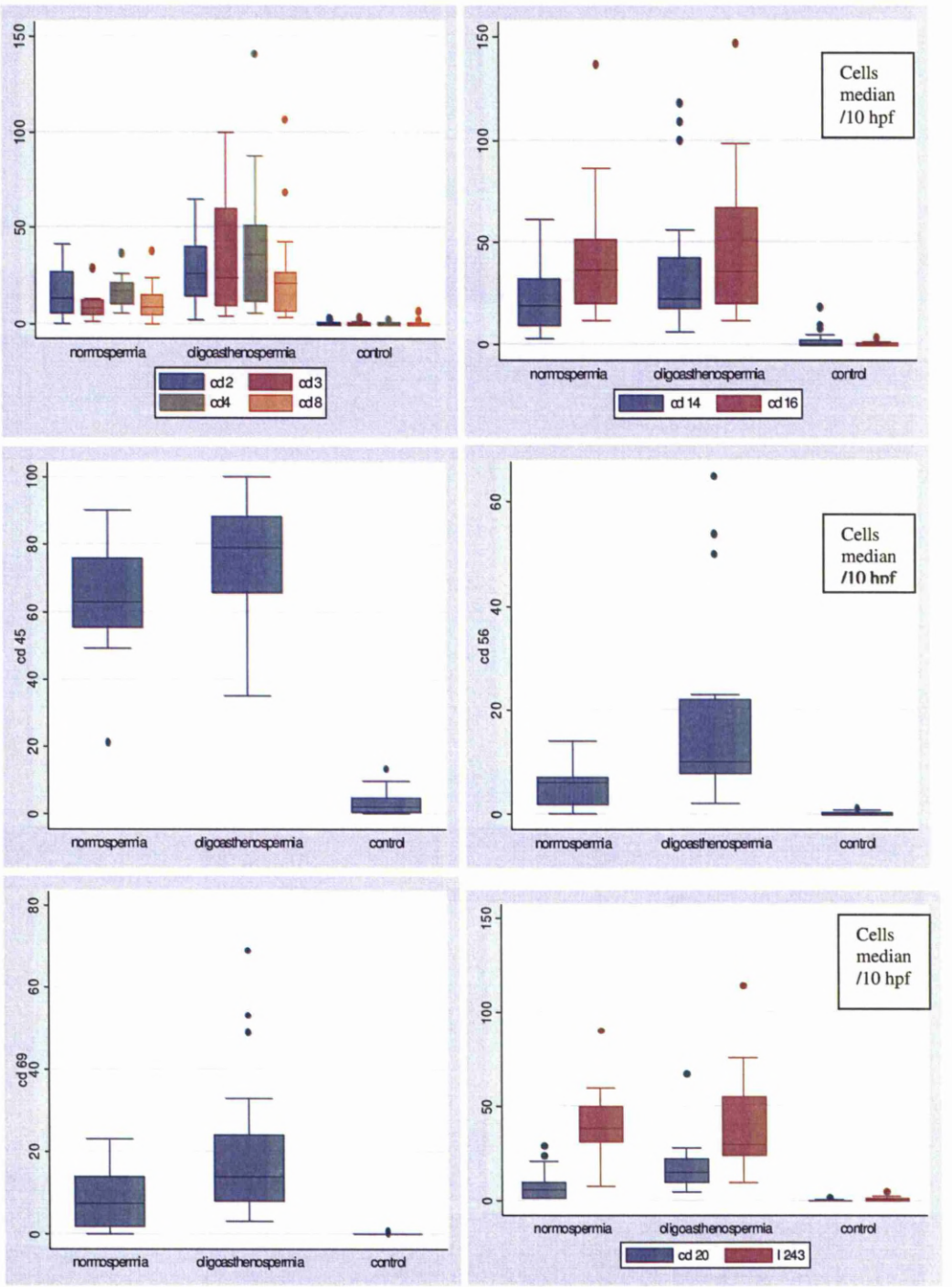


Figure 3.17: Box plots representing the median and ranges of the antibodies (expressed as cells per 10 hpf) in the oligoasthenospermic group vs. the normospermic group and the controls.

A comparison was performed between the oligoasthenospermic group and the control group. The results are outlined in Table 3.16. A representation of the T cells and B cells is shown in Figure 3.17. It can be observed from Table 3.16 that the T and B cells are significantly increased in the oligoasthenospermic group.

The correlations between the sperm parameters and the cell surface markers demonstrate a negative correlation between T cells (CD2 and CD3) with sperm count. Interestingly, there is a negative correlation between CD45 and SMT and morphology as shown in Figure 3.18. It has been noted that increased CD56 levels were associated with decreased sperm count whereas increased CD45 count associated with poor morphology.

There exists a correlation between the T and B cells. There are correlations between the L243 cells and the T and B cells. These correlations are shown in Figures 3.18, 3.19 and 3.20. CD2 correlated with CD20 especially in the severely oligospermic group ($r=0.97$, $p<0.001$). When the mild and the severe oligospermic groups were combined as one group to look for correlations between T and B cells it was found that again CD2, CD3 and CD4 and CD20 correlated well {($r=0.47$, $p<0.04$), ($r=0.48$, $p<0.04$), ($r=0.57$, $p<0.01$)} respectively. However, it was also identified that there were no correlations between CD8 and CD20. Figures 3.21, 3.22 and 3.23 show the L243, CD3 and CD16 cells in the oligoasthenospermic group.

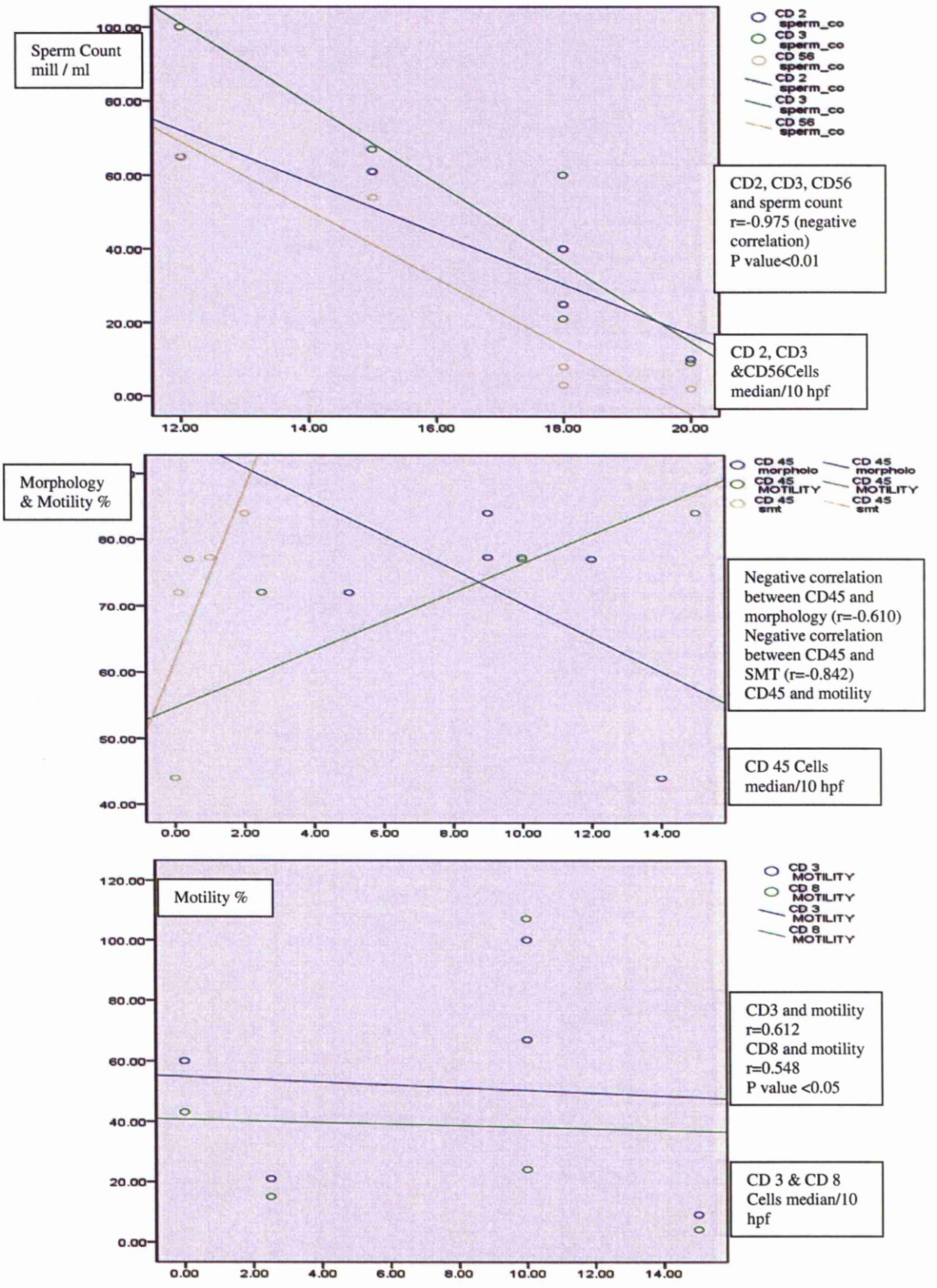


Figure 3.18: Correlation between leucocytes and sperm parameters in the oligoasthenospermic group.

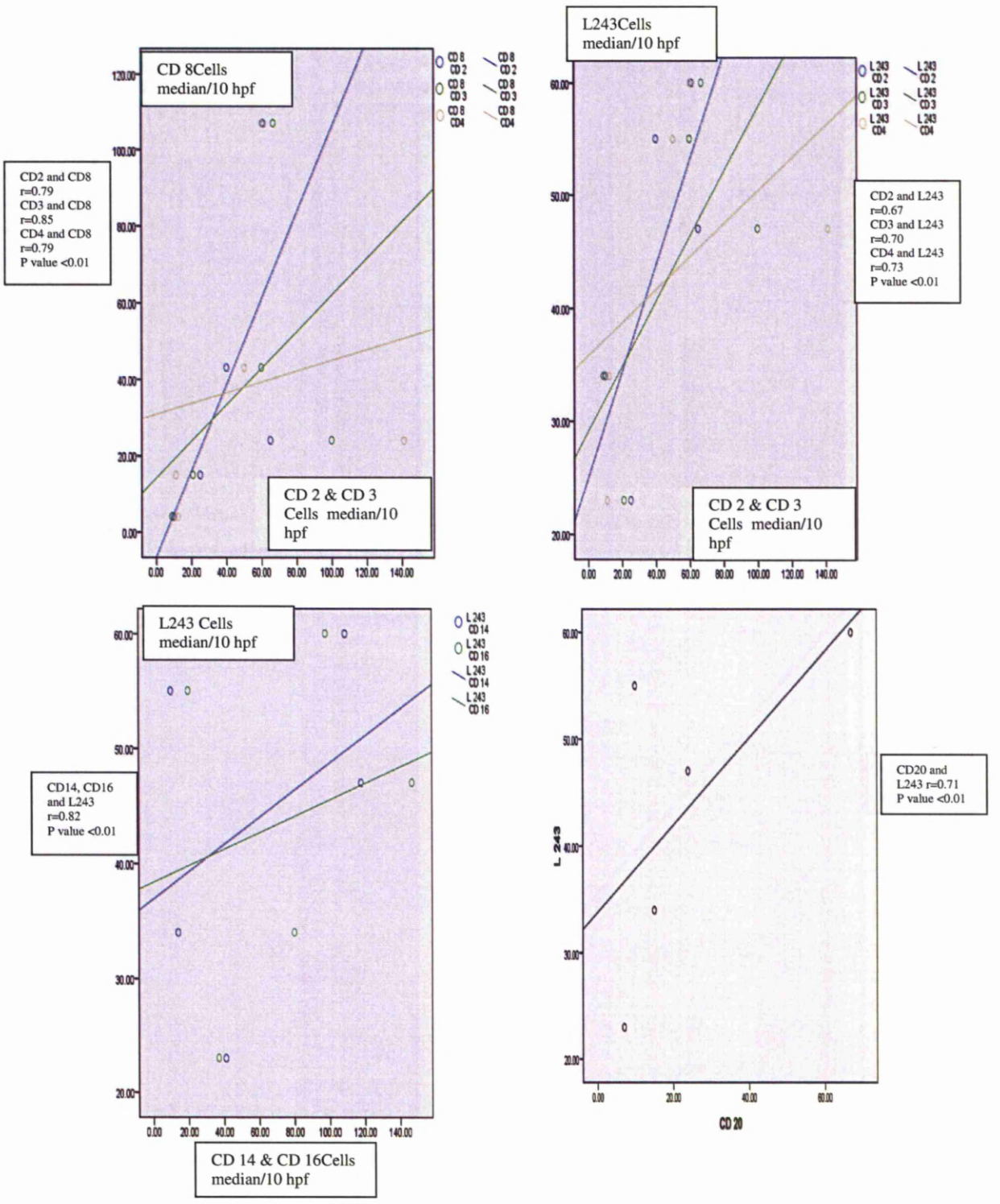


Figure 3.19: Correlations between leucocytes (expressed as cells per 10 hpf) in the mild oligoasthenospermic group.

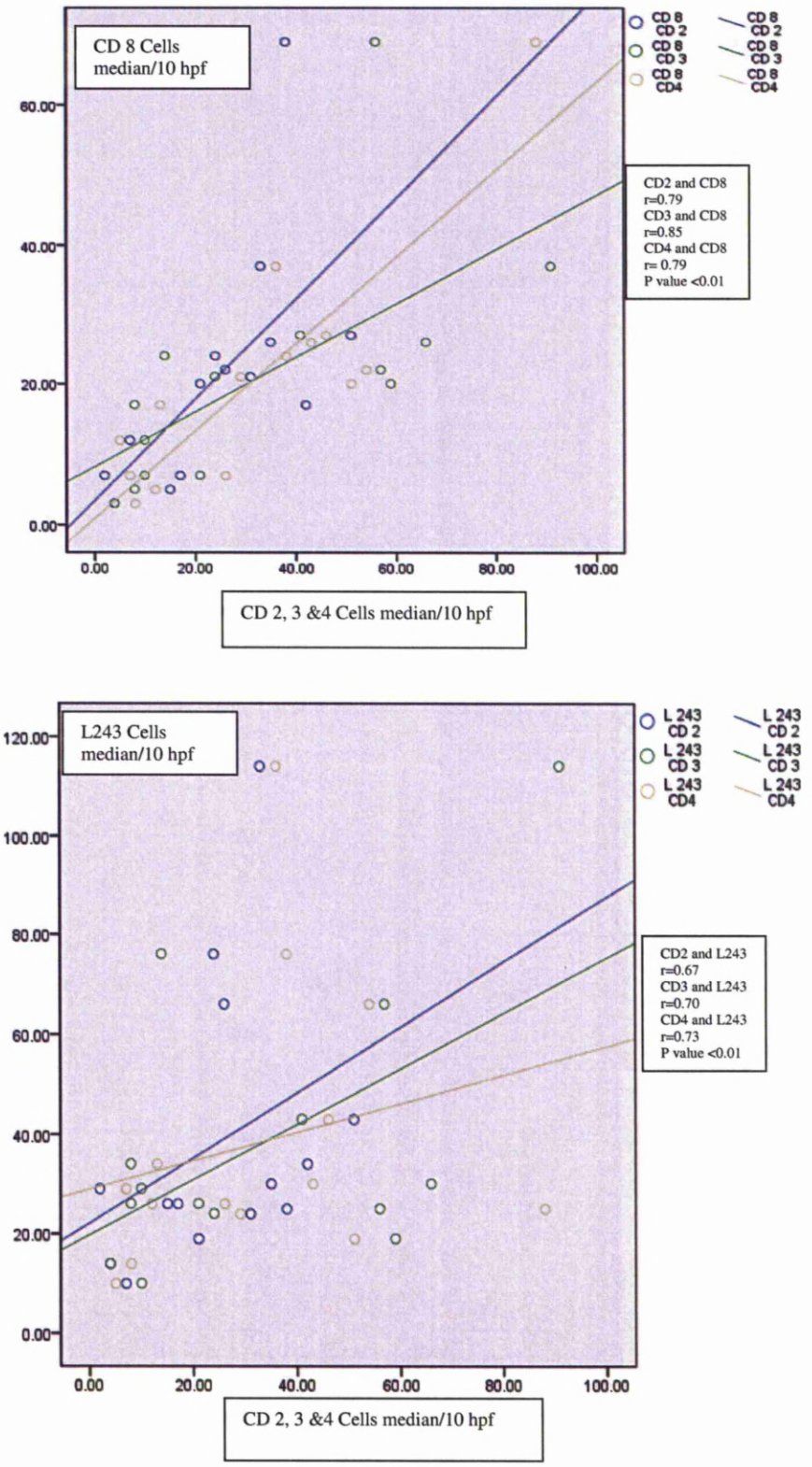


Figure 3.20: Correlation between the leucocytes in the severe oligoasthenospermic group.

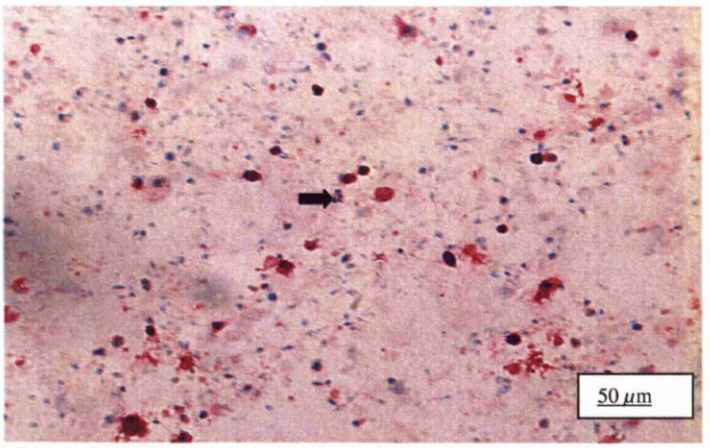


Figure 3.21: L243 cells (stained pink) in the severe oligoasthenospermic sample.

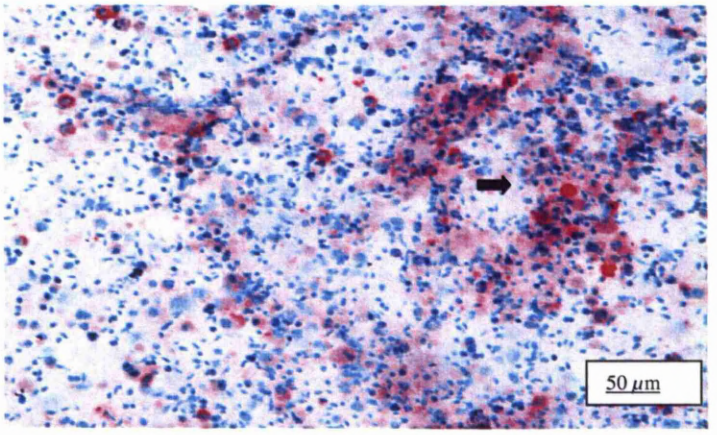


Figure 3.22: CD 3 cells (stained pink) in the severe oligoasthenospermic sample.

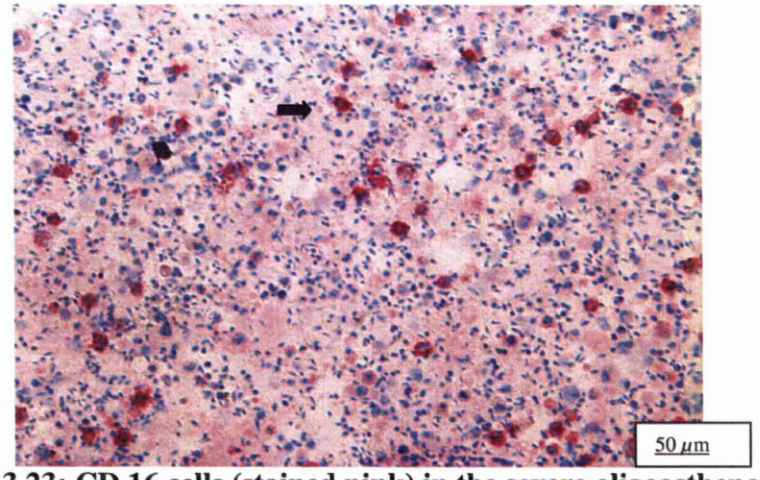


Figure 3.23: CD 16 cells (stained pink) in the severe oligoasthenospermic sample.

3.5.5 Discussion

The results from this section of the study concur with the results of another study where powerful associations were observed between the incidence of leucocytospermia and abnormalities in sperm number and motility[82]. In the severe oligoasthenospermic group, the CD3 and the CD20 levels were significantly elevated. This finding was no surprise and it concurs with the findings of the earlier two studies (studies 3 and 4) where CD3 was elevated in the oligospermic group and CD3 and CD20 were elevated in the asthenospermic group. However, it was also possible to identify an increase in the CD8 and the CD56 cells in the severe oligoasthenospermic group. The CD4 / CD8 ratio was 1.78 in the oligoasthenospermic group when compared to the control group which was 2. This may have some diagnostic utility. It has also been shown that a correlation exists between CD56 (NK cells) and sperm count. The increase in the CD56 count was associated with a decrease in the sperm count. This study identified an increase in the CD45 was associated with poor sperm morphology. In contrast to this study, other studies have failed to identify a significant difference in leucocyte counts between groups of patients exhibiting either a normal semen profile or oligozoospermia[239;240], and even found oligozoospermia to be associated with a significant decline in leucocyte numbers[241]. It has been reported that the presence of abnormal spermatozoa and leucocytospermia can increase ROS production in semen, but their damaging effects on sperm properties are still controversial[107;202;242;243]. According to some authors, ROS generated by leucocytes can be harmful for sperm cells only in the absence of seminal plasma scavenger systems, as in acute testicular or epididymal infections[102]. However, it has been shown here that there exists a balance between T and B lymphocytes on sperm parameters. A disturbance or trigger factor could explain the recruitment of the lymphocytes to enhance sperm damage.

3.6 STUDY NUMBER 6: Leucocyte subsets in the Obstructive Azoospermic and Germ cell damaged Azoospermic group versus the Normospermic group.

3.6.1 Introduction

In the samples with asthenozoospermia, granulocytes were predominant, whereas in those with azoospermia a reduction in the number of macrophages and lymphocytes was observed, suggesting an obstructive process in the azoospermic group, at the level of epididymis and / or vas deferens where these leucocytes are mostly produced. The influence of leucocytes on seminal fluid is controversial, despite its high incidence among infertile men, ranging from 15% to 28%, whereas in fertile men it is 10%[244].

A few studies have failed to detect a negative effect of leucocytes on sperm quality: it has even been suggested that leucocytes have a role in the removal of abnormal spermatozoa from the ejaculate, increasing the percentage of sperm with normal morphology[241], and others have not observed sperm damage during leucocytospermia[245].

On the contrary, many other authors have found evidence that WBCs are a significant cofactor of male infertility, influencing sperm number, motility and functions in a negative way[82;100;225;235], as also demonstrated by the hamster ovum penetration test, an important prognostic factor for IVF[102].

Due to the absence of clinical symptoms, the origin of the leucocytes is unclear. Normally, most leucocytes appear to originate from the epididymis because vasectomised men show very few leucocytes in semen. Sperm damage by WBCs can also be mediated by reactive-oxygen species, proteases and cytokines.

Moreover, it has been suggested that leucocytes induce DNA damage in a cascade-like manner, particularly in sperm with poor morphology and motility[236]. DNA fragmentation also affects the outcome of assisted reproductive technologies. There have been no studies evaluating the presence of leucocytospermia in azoospermic patients.

The aim of the sixth study was to determine which and at what levels the various leucocyte subpopulations are present in azoospermia.

3.6.2 Materials and Methods

Immunohistological staining using monoclonal antibodies was carried out as outlined in Chapter 2 sections 2.5.1, 2.5.2 and 2.5.3.

3.6.3 Statistical Analysis

The analysis was performed as described in Chapter 2 section 2.5.5.

3.6.4 Results

Azoospermia was defined as the complete absence of sperm. Azoospermia was further divided into two groups depending on the aetiology for the azoospermia into either germ cell failure (non obstructive) group or obstructive. The obstructive azoospermic group had all undergone vasectomy.

Although testicular biopsy is the definitive test to rule out the non obstructive from the obstructive groups, the presence or absence of active spermatogenesis can be accurately predicted by measuring the testis volume and serum FSH. Hence the differentiation between germ cell failure azoospermia and obstructive azoospermia was made by the serum follicle stimulating hormone (FSH) levels, karyotyping and testicular volume. The testicular volume in the non obstructive group was reduced (< 7 ml).

20 (Germ cell- 10, Obstructive -10) azoospermic men were identified and compared to 14 normospermic men. In obstructive azoospermia, there was an increase in the number of T cells (CD3; p=0.01), B cells (CD 20; p=0.02), large granular lymphocytes (CD56; p=0.03) and activated T and B cells (CD69; p=0.03) when compared to the normospermic group. These increased numbers were statistically significant (Table 3.17 and Figure 3.24).

All the leucocytes were significantly elevated in the azoospermic group in comparsion to the control group as shown in Figure 3.24. In germ cell damage azoospermia, there was an increase in the number of T cells (CD3; p=0.05). Positive correlation was identified between the leucocytes in the germ cell damage azoospermic group (Figure 3.25). Figure 3.26 shows CD3 cells in a germ cell failure azoospermic sample.

Table 3.17: Median and ranges of the leucocytes in obstructive and germ cell damage azoospermia vs. normospermia. *significance at 5% level **significance at the 1% level.

Antibodies	Normospermic group (n=14) Median and ranges	Obstructive Azoospermia (n=10) Median and ranges	Azoospermia (Germ cell damage) (n=10) Median and ranges	Obstructive Azoospermia vs. normospermic P value	Azoospermia (Germ cell damage) Vs normospermic P value	Azoospermia vs. Normosper mic P value
CD 45%	63(55.5-76)	93(83-97)	80(64-96)	**0.001****	0.1	0.2
CD 45	5.9 (3.9-16.7)	6.65 (3.6-8.4)	5.35 (2.6-11.1)	0.82	0.47	0.57
CD 2	0.7 (0.3-1.9)	1.3 (0.7-2.5)	0.9 (0.5-1.5)	0.45	0.72	0.5
CD 3	0.5 (0.3-1.3)	1.35 (1-2.8)	0.85 (0.6-2.5)	**0.01***	**0.05***	**0.01***
CD 4	1 (0.6-2.2)	2.05 (1.2-3)	1.65 (1.5-3.9)	0.22	0.17	0.12
CD 8	0.6 (0.2-1.1)	1 (0.5-1.7)	0.35 (0.3-2.1)	0.1	0.82	0.26
CD 14	1.2 (0.5-2.8)	2.15 (1.5-3.5)	1.05 (0.7-2.5)	0.11	0.72	0.24
CD 16	2.5 (1.5-5.7)	2.8 (2.2-3.3)	1.7 (1.4-3)	0.96	0.44	0.66
CD 20	0.4 (0.2-1)	1.45 (0.7-3.3)	0.6 (0.3-1.1)	**0.02***	0.33	**0.05***
CD 56	0.4 (0.3-0.5)	0.8 (0.5-1.4)	0.5 (0.1-3.3)	**0.03***	0.47	0.09
CD 69	0.5 (0.2-1.2)	1.6 (1-2.4)	0.65 (0.4-1.6)	**0.03***	0.45	0.08
L243	2.4 (1.7-5.5)	2.5 (1.7-5)	2 (1.3-6.1)	0.96	0.68	0.78
CD4 /CD8 Ratio	1.67	2.05	4.71			

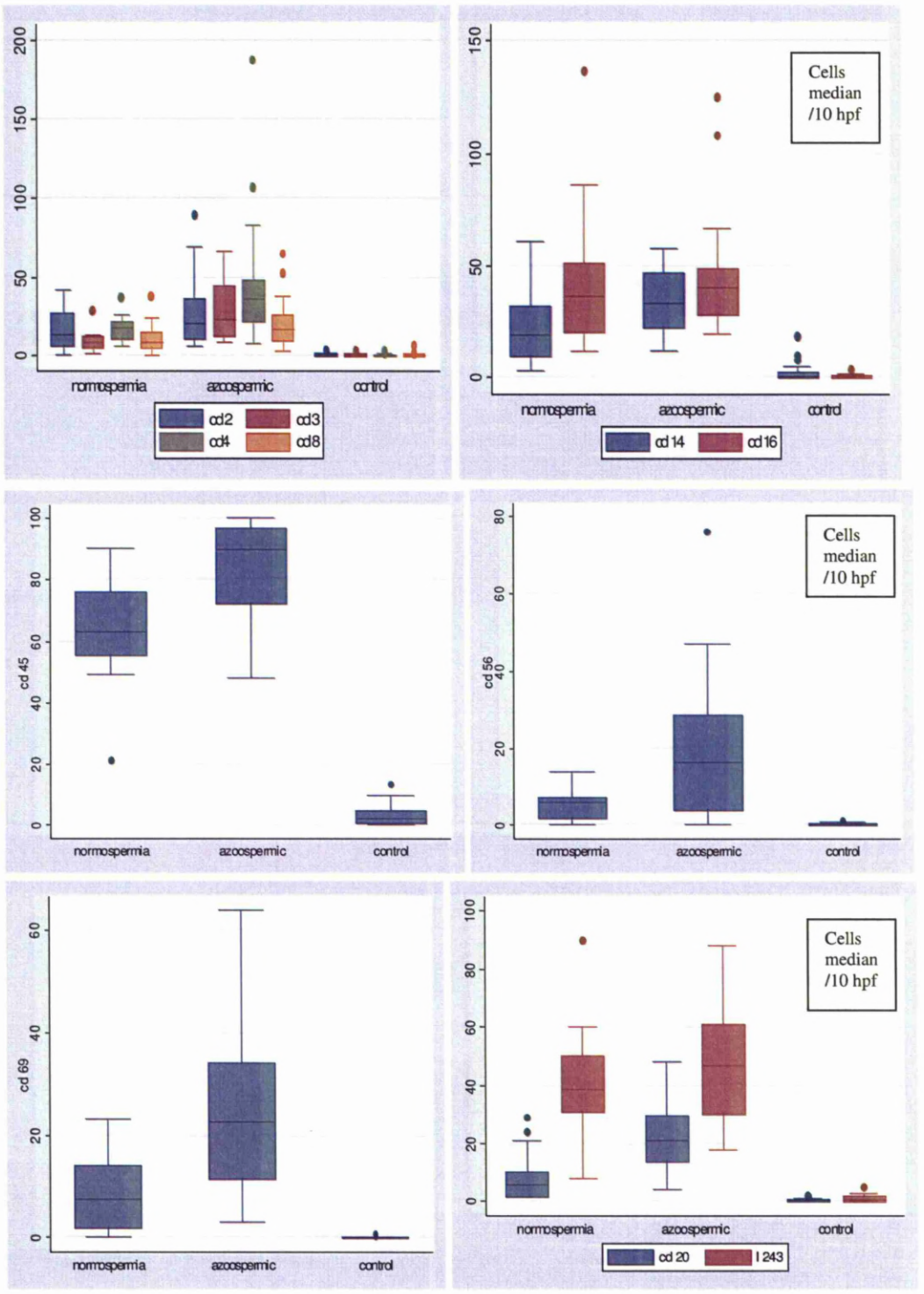


Figure 3.24: Box plots representing the median and ranges of the leucocytes (expressed as cells per 10 hpf) in the azoospermic group, normospermic group and the controls.

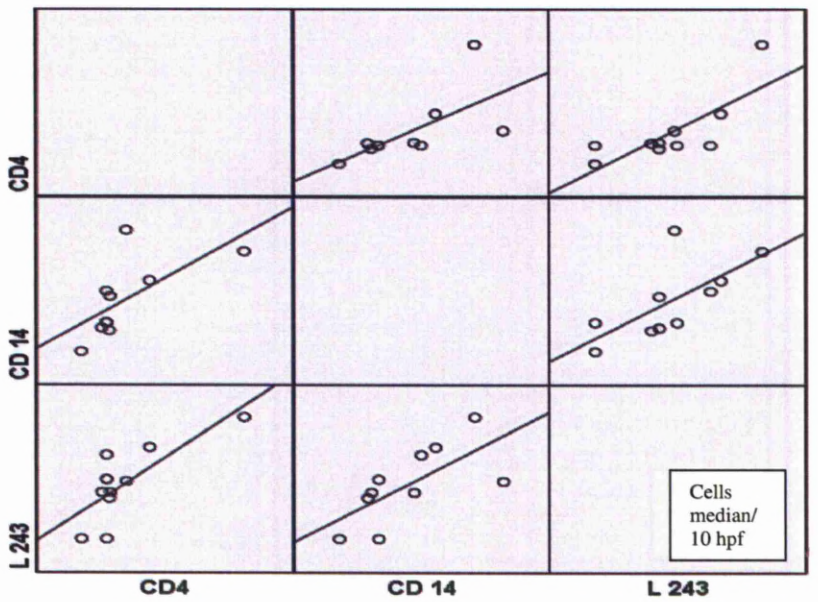


Figure 3.25: Correlation matrix showing the positive correlations (CD4 and L243 r=0.90, CD14 and CD4 r=0.86, CD14 and L243 r=0.97, p values <0.01) between the leucocytes in the azoospermic germ cell failure group.

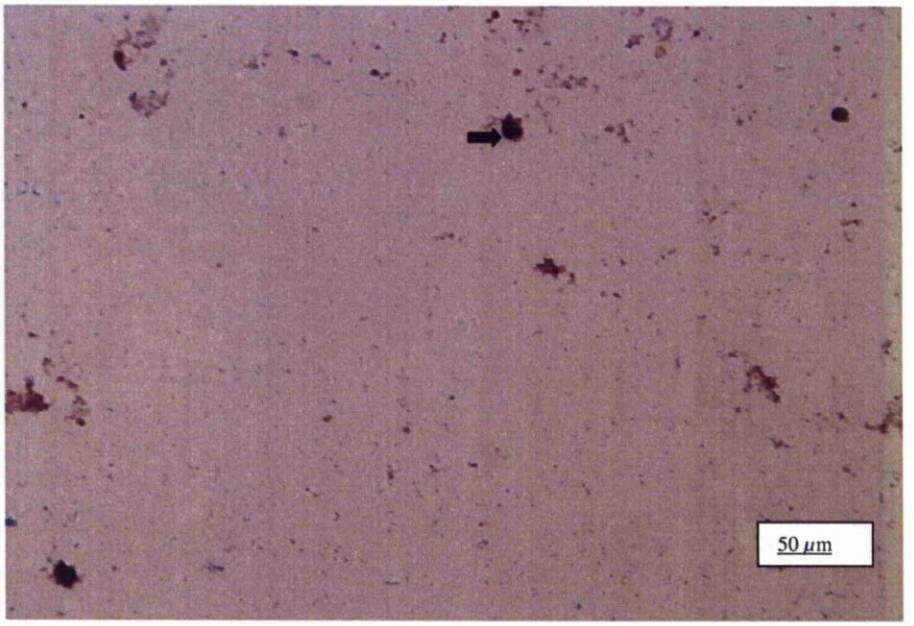


Figure 3.26: CD3 cells (stained pink) in the germ cell failure azoospermic sample.

3.6.5 Discussion

This study has found helper T cells (CD4) as the predominant cell in the control group. The results of this study concur with the findings of a study in rats, where the main T cell population in the epididymis of the rats were the helper T cells[246]. Furthermore, the rat study also showed that in the rat epididymis following vasectomy, there was a decrease in the T4 helper cells number and an increase in the T8 cytotoxic cells number[246]. In direct contrast, a study of the lymphocyte subpopulations in the male genital tract showed that the majority of the T cell population were cytotoxic (CD8) T cells in the scrotal portion of the vas deferens[229]. This ratio changed in patients who had had a vasectomy, where marked increases of helper (CD4) T cells were noted. This study also concurs that there was a significant increase in the cytotoxic cells (CD8) in the obstructive azoospermic group when compared to fertile controls. However, there was also a substantial increase in the helper T cells as well. The CD4 / CD8 ratio in the obstructive group was 2.05 when compared to the germ cell failure group which was significantly raised at 4.71. This ratio might be potentially useful in differentiating between the two azoospermic groups. The seminal population of leucocytes was studied here rather than at the level of the epididymis in the respective groups. In the current study, there was no significant difference in the total leucocyte count or the CD14 (monocyte) count and the two azoospermic groups when compared with normospermic groups as shown in Table 3.18. In addition, the CD3 T lymphocyte and CD56 (large granular lymphocyte) were significantly elevated in the obstructive group. However, all the leucocytes were significantly elevated in the azoospermic group in comparison to the fertile control group.

Lymphocytes, mostly T cells, tend to be concentrated in large numbers in the epididymis, vas deferens and the prostate gland. There were few in number in the rete testis, the ampulla of the vas and the seminal vescicles. B lymphocytes were demonstrated only in the stroma of the

prostate in the same study. Cytotoxic T cells were predominantly present in the lamina propria of the epididymis, vas deferens and seminal vesicles. In contrast, the helper T cells were more abundant in the interstitial connective tissues. It is mostly likely that the cytotoxic T cells in the epithelial lining of the male genital tract function as a major immunological barrier which normally prevents the development of autohumoral or cellular immune responses to sperm antigens[229].

The presence of two predominant T cell subsets in the semen could be as a result of antigen sequestration. The variation in the T cell subset numbers may represent a part of a much wider change that occurs to counteract the increased antigen within the genital tract caused by obstruction of the efferent duct. This could lead to two theories of antigen sequestration by the T cells either by immune sequestration or by immune tolerance. T cytotoxic cells are present in increased numbers as they try to suppress the development of an immune response to the trapped sperm as a result of an obstruction. Similarly, the CD20 and the CD69 counts were significantly elevated in the obstructive group. This increase in the B cell population in the azoospermic group may result in increased levels of antisperm antibody being secreted. This might suggest either the site of seminal leucocyte production is not necessarily confined to the vas or the epididymis as once thought.

The leucocyte subpopulations identified to be significantly increased in this study were CD3 in the germ cell damage azoospermic group and increased CD3, CD20, CD56 and CD69 in the obstructive azoospermic group.

"Detection of subpopulations of leucocytes in different subgroups of semen sample qualities"- This paper has been accepted for publication in Andrologia Journal.

3.7 STUDY NUMBER 7: Leucocyte subsets in the IVF group (Fertilisers versus the Non-Fertilisers)

3.7.1 Introduction

One of the interesting findings of a previous study was the correlation between an increased proportion of sperm with tail defects and the significant increase in sperm ROS production[235]. In standard IVF treatment, men who failed to fertilise had a significantly higher mean proportion of spermatozoa with tail defects in semen than those who were successful fertilisers[247]. In another report, the percentage of sperm with tail defects correlated negatively with fertilisation rates *in vitro* both before and after sperm preparation techniques[248]. Leucocytospermia has been linked with poor fertilisation rates and embryo development rates in the leucocytospermic group compared with control groups although there were no statistical differences for embryo quality, embryo cleavage and pregnancy rates. These results indicate that the outcome of ICSI was negatively affected by the presence of leucocytospermia. In contrast, another study found the overall fertilisation rate and the corresponding pregnancy rates were not affected by the presence of leucocytospermia in patients undergoing IVF / ICSI.

The hypothesis was that leucocytospermia leads to increased ROS production which could induce tail defects in the spermatozoa and thereby affect fertilisation rates. Hence the aim was to determine the true effect of T and B cell subpopulations on the fertilisation rates in an IVF cycle.

3.7.2 Materials and Methods

As outlined in Chapter 2 sections 2.5.1, 2.5.2 and 2.5.3. Semen was obtained from the partners of women attending for IVF cycle, by masturbation after three-five days of sexual

abstinence. The samples were collected and analysed similar to group 1 as described in section 2.2. Couples undergoing traditional IVF only were chosen so as to study the effect of the various leucocyte subpopulations on IVF outcome rather than sperm parameters. The inclusion criteria for the men were that they neither had illnesses nor did they take any medication that could have affected cytokine metabolism. These men did not smoke or drink on a regular basis. These men formed the control group.

3.7.3 Statistical analysis

The analysis was performed as described in Chapter 2 section 2.5.5.

3.7.4 Results

36 men were recruited into the study. The age of the men ranged from 25-45 years (median 34 years). The duration of infertility was a median of 3 years and ranged from 2-6 years. The underlying diagnosis of infertility was due to female factors. All men had a normal sperm count, but their sperm motility ranged from 20–100%. Seven were identified as poor fertilisers (fertilisation rates <35%) and 14 (fertilisation rates >60%) were good fertilisers.

The total leucocyte counts (CD45), between the poor and good fertilisers were not statistically significant. The macrophages and the monocytes (CD14) were significantly elevated in the good fertilisers group (0.7) in comparison to the poor fertilisers (0.3). The correlation of various leucocytes with the fertilising potential is depicted in Figure 3.27. Figure 3.28 depicts the CD45 cells stained pink in the seminal plasma from an IVF sample.

Table 3.18: Median and ranges of the CD counts in men with fertilisation rates < 35% and > 60%.

	Fertilisation rate <35% (n=7) Median and Ranges	Fertilisation rate >60% (n=14) Median and Ranges	P value
CD45	1.8 (0.5-3.9)	2.5 (1.3-5.2)	0.30
CD2	0 (0-0.6)	0.4 (0-0.6)	0.36
CD3	0.1 (0-0.7)	0.25 (0.1-0.6)	0.42
CD4	0 (0-0.3)	0.2 (0-0.4)	0.25
CD8	0 (0-0.2)	0.2 (0.1-0.3)	0.15
CD14	0.3 (0.1-0.5)	0.7 (0.3-1.3)	0.05*
CD16	0.3 (0-1)	0.4 (0.1-0.7)	0.88
CD20	0.1 (0-0.2)	0.15 (0-0.7)	0.28
CD22	0 (0-0.1)	0 (0-0.1)	0.47
CD56	0.1 (0-0.3)	0.15 (0-0.3)	0.51
CD69	0	0.1 (0-0.3)	0.06
L243	1.2 (0.4-1.8)	0.65 (0.4-1.5)	0.59

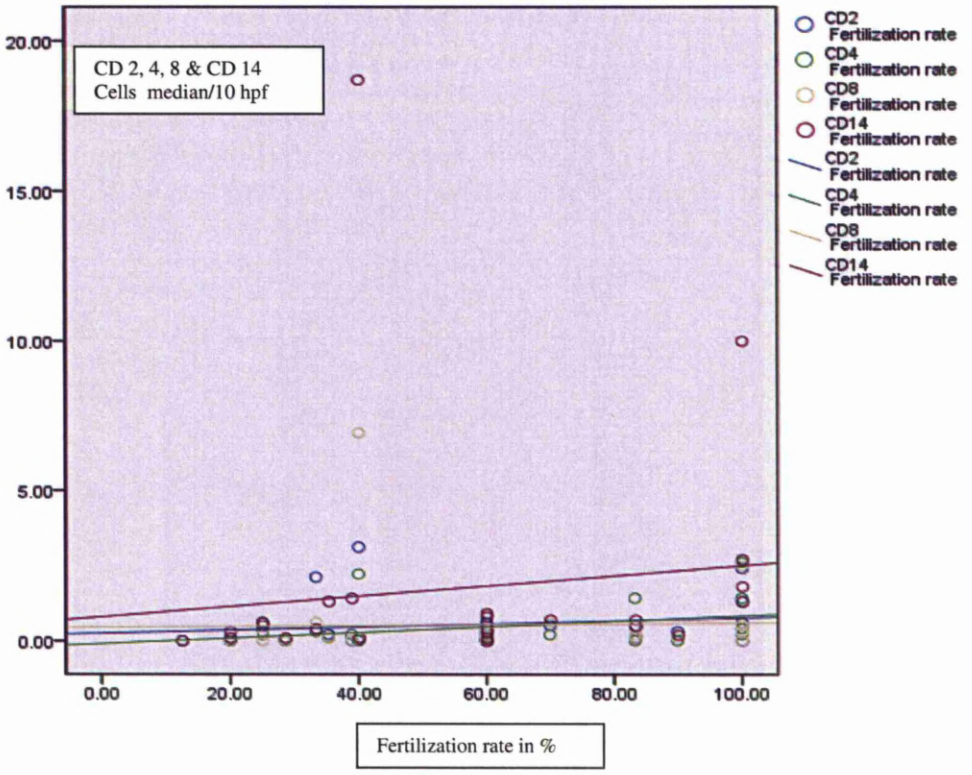


Figure 3.27: Correlation between leucocytes and fertilisation rate showing that the T cells (CD2, CD4, CD 8) and CD14 (macrophages) correlate significantly (r=0.47, p value < 0.01) with the fertilisation rate.

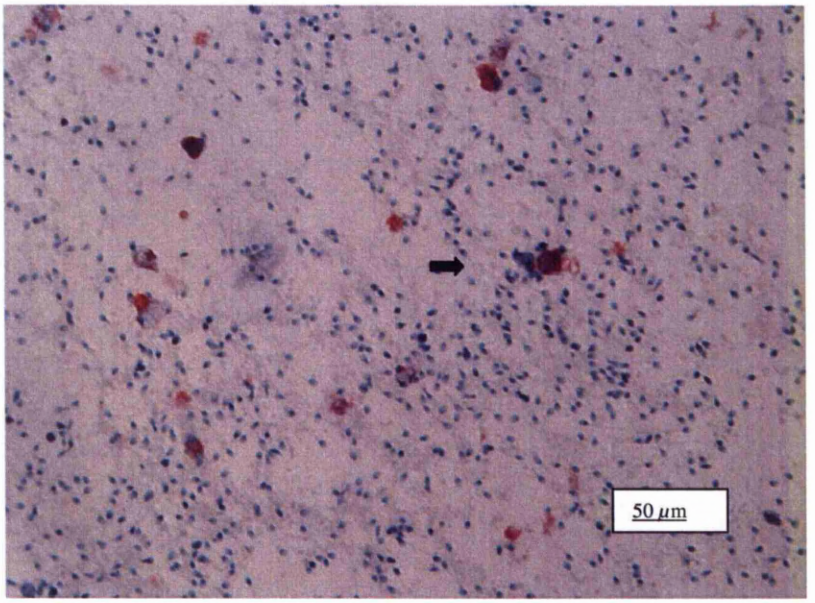


Figure 3.28: CD45 cells (stained pink) in the IVF sample.

3.7.5 Discussion

In this study, the median levels of CD45 were higher in the good fertilisers (2.5) in comparison to the poor fertilisers (1.8). However, this did not reach statistical significance. However, the macrophages and the monocytes (CD14) were significantly elevated in the good fertilisers group in comparison to the poor fertilisers. This could be due to the fact that in these individuals there is increased phagocytic activity which would therefore remove the sperm with abnormal morphology and hence increase the fertilising potential. Another study concurred with this study stating that leucocytospermia may not necessarily have a negative effect on outcome after either *in vitro* fertilisation or intracytoplasmic sperm injection[184]. Similarly, evaluation of the elastase-inhibitor complex in seminal plasma, a marker of male genital tract inflammation, in men undergoing *in vitro* fertilisation (IVF) found no correlation between the seminal elastase-inhibitor levels and fertilisation rates. However, it was shown that leucocytospermia could affect the blastocyst development and lead to embryo arrest[249]. There did not seem to be any role for the T cells in the IVF group.

However, other studies have observed that the concentration of WBC in semen was a strong predictive factor for IVF–embryo transfer success[86;87] and, more recently, it was shown that the number of leucocytes expressing CD45 was negatively correlated with the fertilisation rate of metaphase II oocytes[250]. Similarly, fertilisation rates were found to be significantly reduced in the leucocytospermic group when compared to the control group[183].

These conflicting reports are probably due to different detection methods, different populations studied and to the fact that leucocyte subtypes in semen may have different functions. Most of these studies lacked an accurate, reliable methodology for detecting WBC, making it impossible to draw any firm conclusions from such results. Although

immunocytochemistry is considered the gold standard for the detection of WBC in semen, it is however a manual, subjective method. Data show that correlation between flow cytometry combined with monoclonal antibodies and other methods is limited when used to quantify semen parameters and leucocyte concentrations.

There are a few limitations to this study. The numbers of patients in each group are small. Hence statistical significance may not correlate with clinical significance. Although it has been endeavoured to classify the patient groups as good and poor fertilisers using an arbitrary point of 60% and 35% respectively, some conventional authors may not agree with the quoted reference ranges of semen parameters. It would have also been useful to look at the number of biochemical / clinical pregnancies resulting from the embryos created by the good and poor fertilisers. This would have then led to further discussion about the correlation / relationship between pregnancy outcome and the seminal plasma cytokine content.

Hence the presence of leucocytes does not adversely affect the fertilisation rates and the outcome of an IVF cycle.

CHAPTER 4

CYTOKINES IN THE DIFFERENT SUBGROUPS OF MALE FACTOR INFERTILITY

As discussed in Chapter 1 (section 1.7) cytokines are important mediators of immune and inflammatory reactions. Human semen contains a repertoire of these molecules whose effects on semen quality and sperm function remain the subject of much debate.

This chapter has been divided into subsections. Each of the subsections aims to answer particular questions with regards to cytokines and their role in male subfertility. The objectives were:

4.1- To determine if there is an elevation of cytokines in the ASA+ve patients and whether cytokine mediated sperm damage could explain the subfertility in this group of patients.

4.2- To determine if microbilogically culture positive patients exhibit elevated levels of cytokines and if so whether this could explain their subfertility due to cytokine mediated sperm damage.

4.3- To determime the presence of a repertoire of seminal cytokines IL-6, IL-8, IL-10, IL-11, IL-12, TNF-α, and IFN-γ and to evaluate their role in male subfertility and identify any possible networks that may exist.

4.4- To determine the presence of a network of cytokines within the seminal plasma and whether this network may influence fertilisation rates either directly or indirectly.

4.1 STUDY NUMBER 1: Cytokines in Antisperm Antibody Positive versus Antisperm Antibody Negative patients versus the Controls

4.1.1 Introduction

Immune responses, both humoral (ASA) and cell mediated (CMI), against sperm may be implicated in some cases of infertility of undefined aetiology and in male factor infertility. A study has suggested that a high IL-8 and IL-6 concentration occurred more frequently in ejaculates with IgG class ASA, but this did not reach statistical significance[251].

There was no relationship of TNF-α and IL-1β levels in seminal plasma with semen quality or parameters of sperm functional capacity, and there was no association with local ASA of the IgG or IgA class in another study[252]. However, IL-12 was detected at significantly lower levels in both male factor infertile and immunoinfertile men[253].

Some studies have shown an increase in IFN-γ and IL-6 levels in seminal plasma of infertile / immunoinfertile men compared to those of fertile men[254;255]. The main aim of this study was to show if elevation of cytokines in the ASA+ve patients could explain their subfertility as a result of cytokine mediated sperm damage.

4.1.2 Materials and Methods

The cytokine assays were performed using the cytokine sandwich ELISA method as outlined in Chapter 2 section 2.6.2.

4.1.3 Statistical Analysis

The analysis was performed as described in Chapter 2 section 2.6.3.

4.1.4 Results

64 patients were recruited into this study out of which 26 fertile men formed the control group. These 26 men were attending the IVF clinic due to female factor infertility, were ASA-ve, culture negative and their partners had achieved a pregnancy and thus were considered to be an adequate control group. IL-10 was significantly raised in the ASA+ve group when compared to the control group (Table 4.1).

However, there was no significant difference between the ASA+ve and the ASA-ve group. The cytokines to reach statistical significance in the ASA+ve and ASA-ve group when compared to the control group were IFN-γ and TNF-α as shown in Table 4.1. In addition, IFN-γ was also found to have a negative correlation with sperm motility as shown in Figure 4.1.

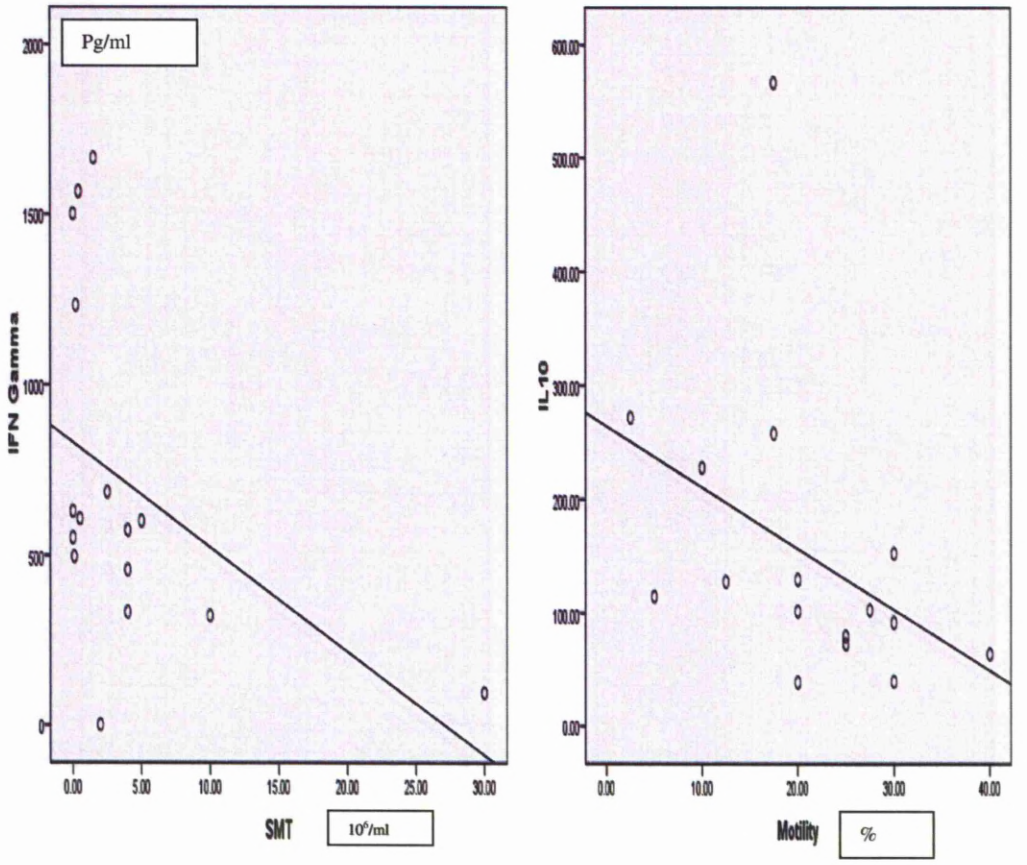


Figure 4.1: Effect of cytokines (pg/ml) on motility and SMT- A negative correlation (IFN gamma and SMT r=-0.525, IL-10 and motility r=-0.310, p value< 0.01).

Table 4.1: Comparison of the cytokine levels between each subject group. Values are expressed as picograms/ml, with ranges for each group given in brackets. P value indicates that results reached statistical significance between the following comparisons: ASA negative vs. controls, ASA positive vs. controls, ASA negative vs. ASA positive. *Significant at 5% level **significant at 1% level.

Cytokines pg/ml	ASA Negative (n=21) Median and Ranges	ASA Positive (n=17) Median and Ranges	Controls (n=26) Median and Ranges	P –value Negative vs. controls	P –value Positive vs. controls	P –value Positive vs. negative
IL-6	21.5 (13-60.5)	40 (21-84.5)	35 (24-56)	0.31	0.58	0.19
IL-8	1400 (950-1700)	1800 (1350-2100)	945 (320-1800)	0.54	0.06	0.25
IL-11	6370 (2550-9460)	5365 (2500-9600)	3175 (2317-5475)	0.16	0.09	0.96
IL-10	83.5 (63.5-145)	109 (76.5-190.5)	61.67 (35.83-100)	0.27	0.02*	0.28
IL-12	11.5 (3-31.75)	6 (0-15)	12 (7-15)	0.49	0.23	0.14
TNF-Alpha	0	0	8 (0-18)	0.11	0.01**	0.63
IFN-Gamma	605 (390.5-955)	587.5 (396-958.5)	83 (42-329)	0.0001**	0.0001**	0.85

4.1.5 Discussion

Mechanisms that result in ASA production and autoimmunity are enhanced by the secretion of proinflammatory cytokines such as IL-1, IFN-γ, TNF-α, and reduced by the secretion of antiinflammatory cytokines such as IL-10[256;257].

In this study, statistically significant raised levels of IL-10 ($p<0.02$) were found in the ASA+ve group when compared to the control group which may support the view that IL-10 is an antiinflammatory cytokine with immunoregulatory functions and inhibits the production of several proinflammatory cytokines including TNF-α and IFN-γ by altering the accessory cell function of macrophages. Hence the increased levels in the ASA+ve group compared to the ASA-ve group. In agreement with previous studies[252], no relationship between TNF-α and antisperm antibodies was found.

Significant levels of IFN-γ were present in the subfertile group irrespective of their ASA status when compared to the control group. IFN-γ was also found to have a negative correlation with the SMT as shown in Figure 4.1. IFN-γ, at high concentrations, has been noted to adversely affect sperm motility[200] and men with antisperm antibodies have lower sperm motility[201] such that the mechanism of the effect of antisperm antibodies on sperm motility may be via the action of IFN-γ.

These findings support the hypothesis that there are significant differences in the levels of certain cytokines in the ASA+ve patients. Some have a protective role (IL-10 the antiinflammatory cytokine was significantly raised in the ASA+ve group), whereas others can cause subfertility through cytokine mediated damage which seems to particularly affect

sperm motility (raised IFN-γ in the subfertile group irrespective of the ASA status). This could explain that previous theories of how ASA could affect sperm motility in a mechanical way by antibody binding to the sperm tail may not be wholly true and the cytokine IFN-γ may have had a role to play.

4.2 STUDY NUMBER 2: Cytokines in patients with Positive cultures versus patients with Negative cultures

4.2.1 Introduction

Inflammatory cytokines are produced by white blood cells (WBC), mainly by macrophages, in response to foreign antigens, pathogens (infection challenge) and also in chronic inflammation (immunological activation). Acute and chronic infections may play a contributory role in male infertility. The clinical relevance of silent infection in asymptomatic patients is, however, not clear. Cytokines play an important role in the immuno-inflammatory mechanisms which underlie the host response to infection, the most appropriate subdivisions being those that modulate leucocytes to produce proinflammatory responses, and those that have the capacity to down regulate inflammatory cells[172].

The appearance of different cytokines in relation to infection might depend upon the infecting organism and the genetic make up of the individual[258]. It was hypothesised that even subclinical or asymptomatic infection could mount an immune response thereby causing an elevation of cytokines in the seminal plasma. Therefore, the aim of the study was to identify if elevated levels of cytokines in the culture positive patients could explain their subfertility due to cytokine mediated sperm damage.

4.2.2 Materials and Methods

The cytokine assays were performed using the cytokine sandwich ELISA method as outlined in Chapter 2 section 2.6.2.

4.2.3 Statistical Analysis

The analysis was performed as outlined in Chapter 2 section 2.6.3.

4.2.4 Results

The total number of men recruited was 76 for the cytokine study. Three samples were lost during transport for cytokine analysis and hence the final study number was 73. In this study there were no significant differences in the levels of cytokines (IL-6, IL-8, IL-10, IL-11, IL-12, TNF-α, and IFN-γ) between the culture positive and the culture negative groups. This was true not only for the *Ureaplasma* group as shown in Table 4.2, but also for the general culture positive group which had organisms such as *Enterococcus*, mixed anaerobes, *Streptococcus milleri* and beta haemolytic *Streptococcus* as shown in Table 4.3.

In the *Ureaplasma* culture positive group there was a highly significant negative correlation ($p< 0.01$) between IL-11 and sperm parameters (morphology, motility and SMT) as shown in Figure 4.2. There was also a significant negative correlation ($p<0.05$) between IL-10 and SMT as shown in Figure 4.2. In the general culture positive group there was a highly significant negative correlation between IL-11 and sperm morphology ($p<0.01$) and an equally significant negative correlation between IFN-γ and sperm morphology and sperm count as shown in Figure 4.3. Similarly, IL-10 had a significant negative correlation to sperm count, morphology and SMT in the general culture positive group as shown in Figure 4.4.

Table 4.2: Median and ranges of cytokines (pg/ml) in Ureaplasma positive vs. Ureaplasma negative groups.

Cytokines pg/ml	Positive (n=20) Median and Ranges	Negative (n=53) Median and Ranges	P –value Positive vs. negative
IL-6	36.5 (22-84.5)	30 (18-69)	0.5
IL-8	1950 (1400-2600)	1750 (1375-2500)	0.84
IL-10	104.5 (65-172.5)	117 (80-202)	0.53
IL-11	5500 (4083-9250)	6579 (3700-11670)	0.72
IL-12	4.5 (0-20.5)	13.5 (0-40)	0.18
TNF alpha	0	0 (0-6.5)	0.49
IFN gamma	461 (313.5-1069)	741 (456-1200)	0.09

Table 4.3: Median and ranges of cytokines (pg/ml) in general culture positive vs. negative. (Culture positives included Enterococcus, Mixed Anaerobes, Streptococcus milleri and Beta haemolytic streptococcus).

Cytokines pg/ml	Positive (n=24) Median and Ranges	Negative (n=46) Median and Ranges	P –value Positive vs. negative
IL-6	24 (17-50)	38 (19-96)	0.08
IL-8	1720 (1000-2100)	1950 (1450-2600)	0.11
IL-10	117 (73-175)	112 (70-210)	0.89
IL-11	6579 (4150-11500)	6130 (3167-9983)	0.62
IL-12	13 (0-72)	9 (0-25)	0.33
TNF-alpha	0 (0-20)	0	0.1
IFN-Gamma	744 (328-1429)	656 (347-1109)	0.53

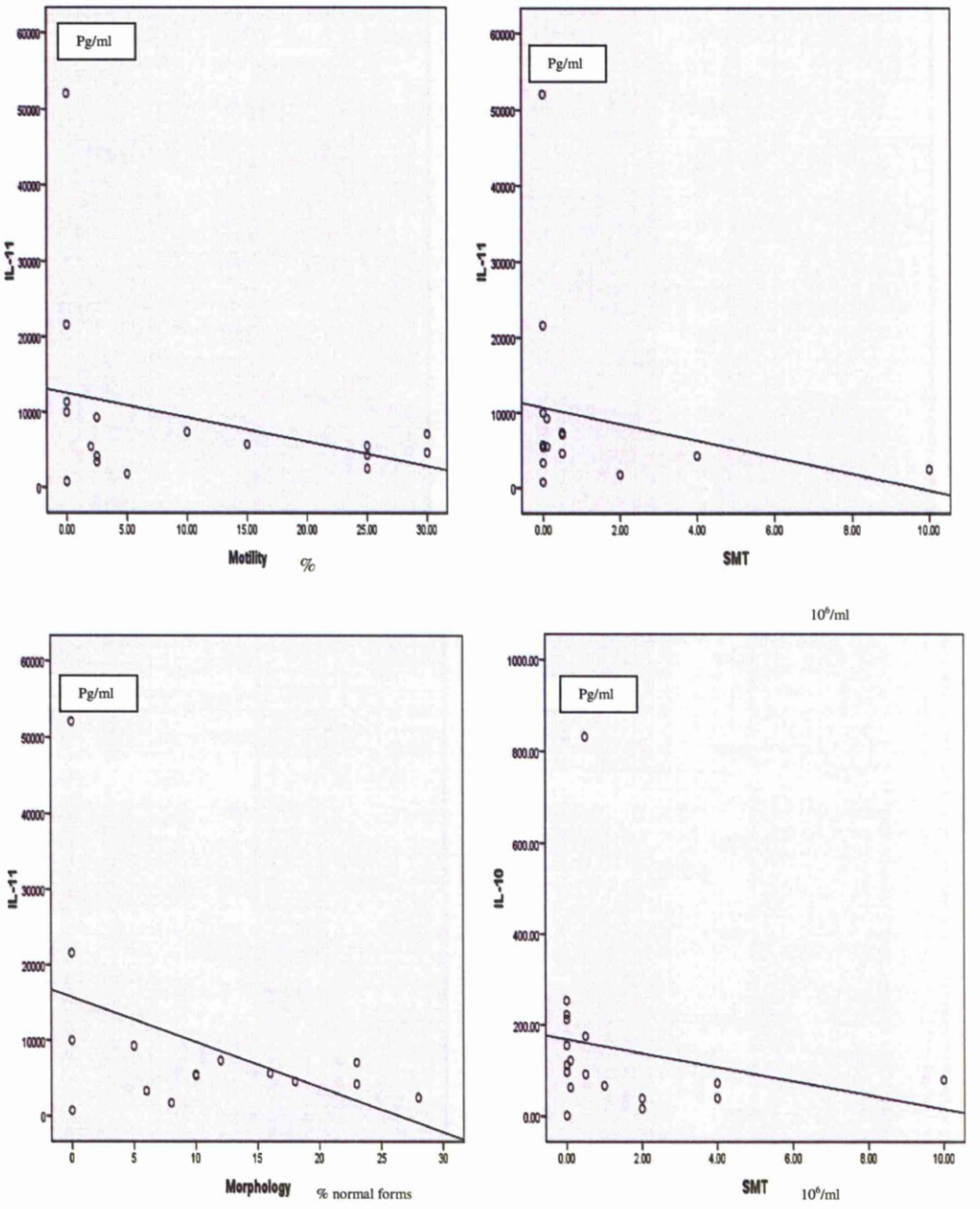


Figure 4.2: Negative correlation between cytokines and sperm parameters in the Ureaplasma positive group (IL-11 and motility and SMT r=-0.27, IL-10 and SMT r=-0.252, IL-11 and morphology r=-0.296, p value< 0.01).

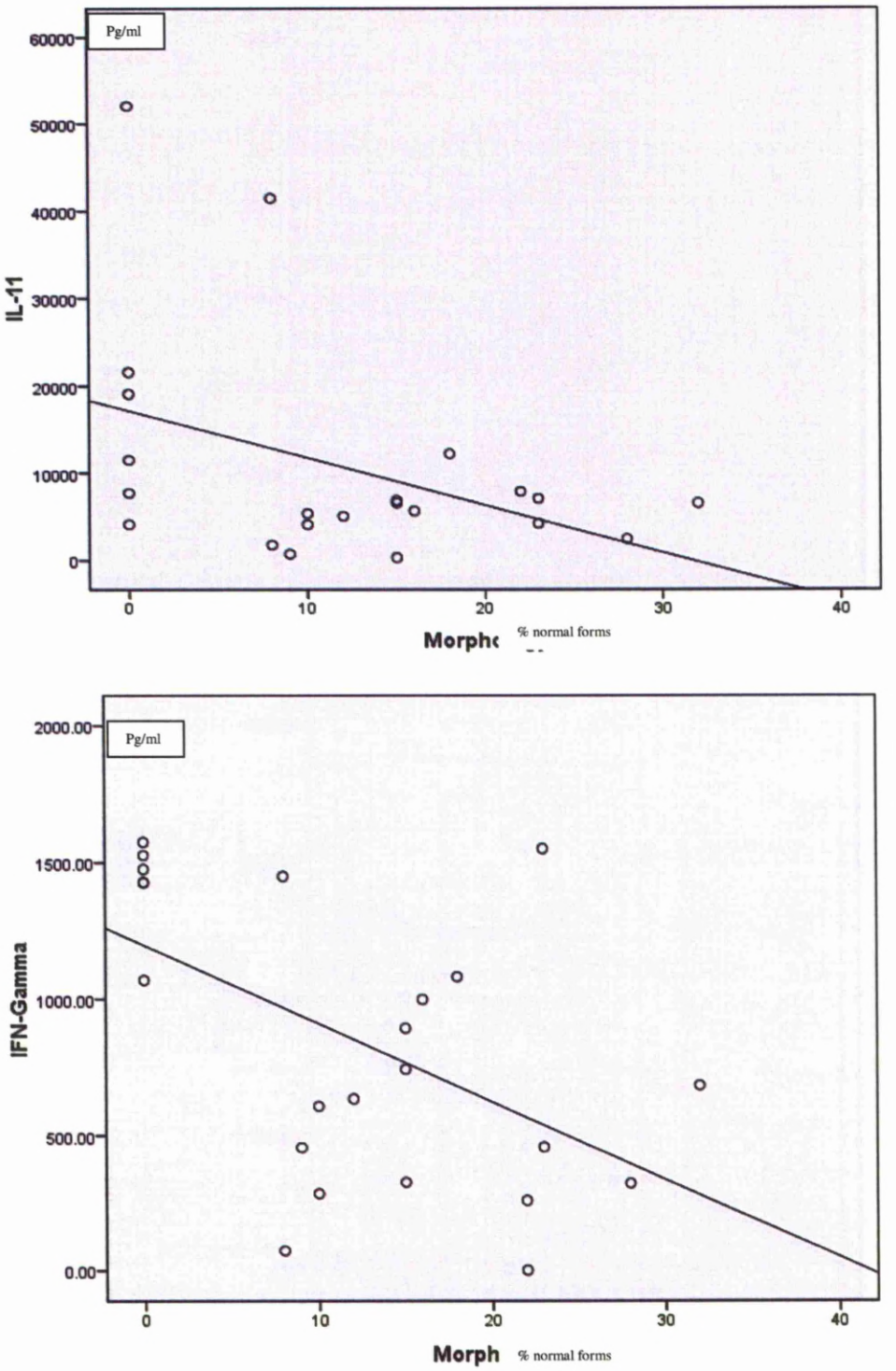


Figure 4.3: Negative correlation between IL-11 and IFN- gamma with sperm parameters in the general culture positive group (IL-11 and morphology r=-0.296, IFN- gamma and morphology r= -0.476, p value< 0.01).

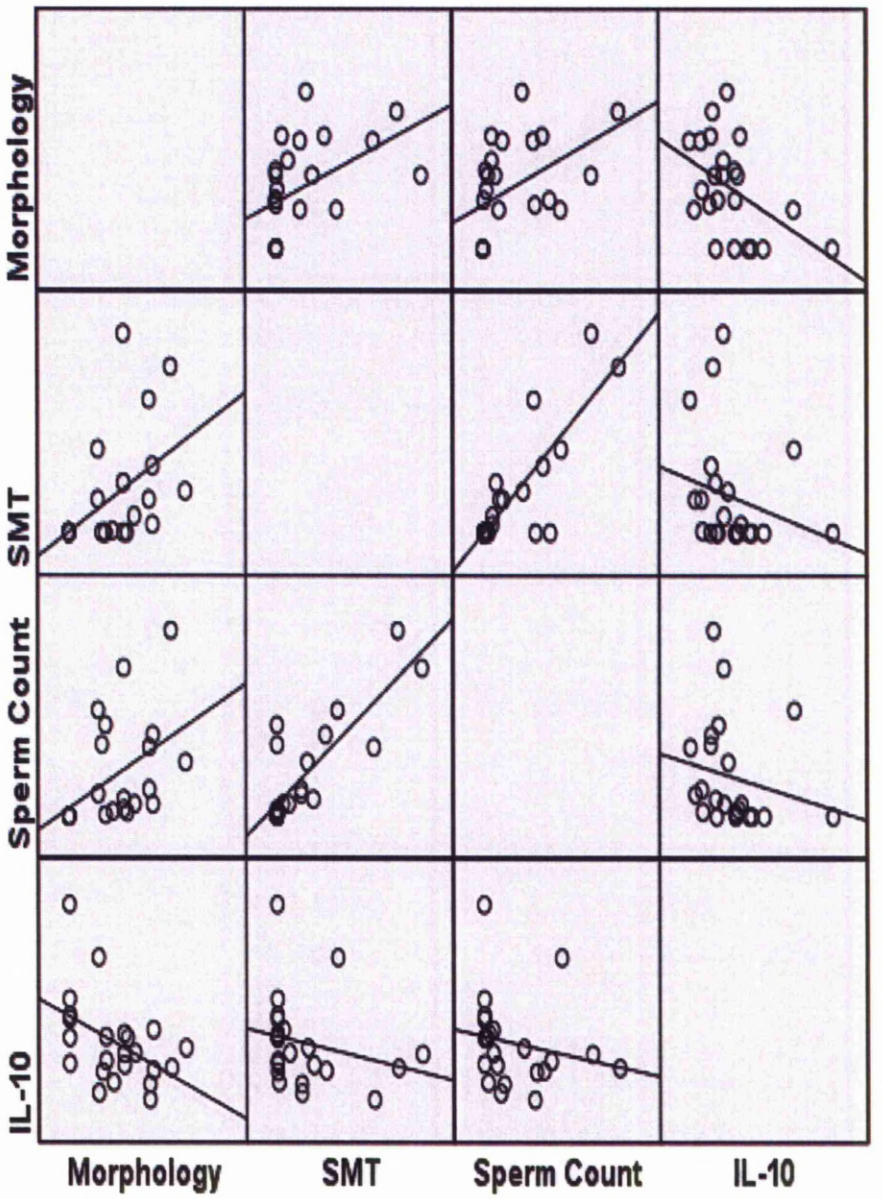


Figure 4.4: Correlation matrix depicting the relationship between IL-10 and sperm parameters in the general culture positive group (IL-10 (pg/ml) with SMT (10^6/ml), sperm count (10^6/ml) and morphology (% normal forms) r=0.386, 0.362 and 0.343 respectively, p value < 0.01).

4.2.5 Discussion

There was no significant difference in the IL-6 levels between the two groups in this study. This finding is in agreement with another study by Kocak et al. which showed no significant difference in IL-6 levels between normal men and those with male accessory gland infections (MAGI)[259]. This may be due to the wide range of values for proinflammatory cytokines in the MAGI group.

IL-8 is a chemokine and has been thought to play a key role in T cell mediated immune responses. There was no significant difference detected between the levels of IL-8 in the culture positive or culture negative group in this study. The origin of IL-8 in the male reproductive tract and its role in spermatogenesis and steroidogenesis has not been elucidated but a study had shown that there were significant differences in levels between normal men and infertile men with leucospermia / male accessory gland infection[260]. An increased IL-8 secretion was shown in the infertile group with infection of the accessory genital glands[261]. The findings of the above two studies are in direct contrast to this study. This could be due to the fact that the patients in the before mentioned studies had overt infection compared to this patient group who had only subclinical infection.

There was no significant difference between the IL-10 and IL-11 levels amongst the two groups, but IL-11 had a significantly negative correlation with sperm parameters in the culture positive group. Although increased levels of IL-11 were found in men with male accessory gland infection when compared to normal men, IL-11 levels did not show any significant difference between normal men and those with abnormal spermiograms[262]. However, it is likely that rather than acting independently, IL-11 acts in combination with IL-6 and IL-8 in affecting sperm parameters particularly in patients with urogenital infections.

Thus the presence of IL-11 in seminal plasma may still be considered as a potential marker of infertility.

Unfortunately, it is not possible to form any conclusions on the role of Chlamydial infection and sperm parameters and cytokine levels in the seminal plasma as none of the patients studied tested positive for *Chlamydia*. It could be argued that one of the potential weaknesses of this study was the non utilisation of molecular laboratory techniques to identify Chlamydial infection. Similarly, this patient group did not test positive for *Mycoplasma species* or *Trichomonas vaginalis*. Direct culture of semen is ideally the best practice method to detect male genital tract infection as it is 100% specific. However, these are technically demanding, needing a cold chain particularly for *Chlamydia* to preserve specimen viability in transport and are not readily available. Furthermore, the results can take up to eight days. Nucleic Acid Amplification Tests (NAAT) are both highly sensitive and specific (95%). The results of NAAT are received within a few hours. Thus, NAAT is replacing culture as the tests of choice in detecting male genital tract infections. However, to date there are no commercial NAAT semen tests available[263]. Furthermore, men attending an infertility clinic are likely to have reduced sperm quality which could have had an impact on the findings here.

There were no significantly elevated cytokine levels in the culture positive group and hence it is difficult to solely implicate cytokines as the cause of apparent subfertility in this group. This would go to suggest that the presence of asymptomatic infection might not produce the desired inflammatory response including the production of cytokines or that the distal urogenital tract is not sterile. Not many cytokines had significant negative correlations with

sperm parameters which could suggest that the cytokines may not be originating in the testis and thus may not be influencing spermatogenesis.

Due to the lack of positive patients in this study, it is impossible to either support or refute the role played by *Chlamydia trachomatis, Mycoplasma species* and *Trichomonas vaginalis* infections in male subfertility.

4.3 STUDY NUMBER 3: Cytokines in different subfertile groups

4.3.1 Introduction

During the last decade there has been much evidence to suggest the involvement of paracrine control mechanisms such as the cytokines, in the control of reproductive function, particularly with regard to spermatogenesis. Cytokines may have a multitude of actions including both as growth and differentiation factors within the seminiferous tubule. It was hypothesised that there exists an intricate network of cytokines within the seminal plasma which may influence sperm function directly or indirectly. Thus the main aim of the study was to evaluate the role of a repertoire of seminal cytokines IL-6, IL-8, IL-10, IL-11, IL-12, TNF-α, and IFN-γ in male subfertility and identify any possible networks that may exist.

4.3.2 Materials and Methods

The cytokine assays were performed using the cytokine sandwich ELISA method as outlined in Chapter 2 section 2.6.2.

4.3.3 Statistical Analysis

The analysis was performed as outlined in Chapter 2 section 2.6.3.

4.3.4 Results

The total number of men recruited was 76 for the cytokine study. Three samples were lost during transport for cytokine analysis and hence the final study number was 73. The normospermic group was compared with each of the other patient groups. The number recruited for the study is shown in Table 4.4.

The groups were defined as:

1. Normospermia.
2. Asthenospermia- sperm count $>20x10^6$ and a motility of <40%.
3. Mild oligospermia - $10\text{-}20x10^6$ and >40%.
4. Severe oligospermia - $<10x10^6$ and >40%.
5. Mild oligoasthenospermia - $10\text{-}20x10^6$ and <40%.
6. Severe oligoasthenospermia - $<10\ x10^6$ and <30%.
7. Azoospermia - complete absence of sperm in the semen.

Table 4.4: The groups of men recruited into the study

Patient Groups	Cytokine Study
Normospermia	14
Oligospermia	13
Asthenospermia	8
Oligoasthenospermia	19
Azoospermia (Obstruction)	10
Azoospermia (GCF)	9

Azoospermia was further divided into two groups depending on the aetiology for the azoospermia into either germ cell failure (non obstructive) group or obstructive. The obstructive azoospermic group had all undergone vasectomy. Although testicular biopsy is the definitive test to rule out the non obstructive from the obstructive groups, the presence or absence of active spermatogenesis can be accurately predicted by measuring the testis volume and serum FSH. Hence the differentiation between germ cell failure azoospermia and obstructive azoospermia was made by the serum follicle stimulating hormone (FSH) levels, karyotyping and testicular volume. The testicular volume in the non obstructive group was reduced (< 7 ml).

The median values and the ranges for all the cytokines are given in Table 4.5. Significantly higher concentrations of IL-6 ($p<0.05$) were present in the asthenospermic, mild oligospermic, severe oligospermic, severe oligoasthenospermic, azoospermic obstructive and germ cell damage groups when compared to the normospermic group. Significantly higher concentrations of IL-8 ($p<0.05$), were present in the asthenospermic group when compared to the normospermic group. Significantly higher concentrations of IL-10 ($p<0.05$), were present in the asthenospermic, severe oligoasthenospermic and azoospermic obstructive group when compared to the normospermic group as shown in Figure 4.5.

Table 4.5: The median and ranges of cytokine levels (pg/ml) in the normospermic group are compared to the different subsets of subfertile men (*= p<0.05).

Cytokine levels Pg/ml	IL-6	IL-8	IL-10	IL-11	IL-12	TNF-α	IFN-γ
Normospermic (n=14)	18 (4-73)	1300 (220-2600)	76.5 (0-357)	6472 (13-11760)	21.5 (0-100)	0 (0-44)	454 (97-2135)
Asthenospermic (n=8)	69.5 (4-568)*	2850 (450-49500)*	158 (35-832)*	4602.5 (750-27417)	11 (0-26)	0 (0-0)	563 (192-1665)
Mild oligospermic (n=6)	46 (16-166)*	1400 (25-2500)	85 (39-153)	9820 (6130-14083)	6.5 (0-25)	0 (0-41)	561.5 (2-1232)
Severe oligospermic (n=7)	26 (8-140)*	1650 (1400-1750)	78 (43-175)	5083 (2550-12210)	15 (0-72)	0 (0-20)	636 (314-1551)
Mild oligo astheno spermic (n=5)	19 (8-355)	2100 (320-2600)	58 (25-245)	5850 (667-9250)	0 (0-55)	6 (0-30)	723 (224-1109)
Severe oligoastheno spermic (n=14)	33.5 (7-211)*	1675 (160-32750)	137.5 (0-272)*	4104 (333-16980)	0 (0-110)	0 (0-51)	619 (59-1566)
Azoospermic obstructive (n=10)	42 (14-101) *	2050 (1250-5000)	186 (80-490)*	9529 (3750-21804)	25 (6-140)	23 (0-73)*	1049.5 (202-1704)*
Azoospermic non obstructive (n=9)	37 (11-1006)*	2100 (700-3250)	93 (2-253)	4991.5 (0-33441)	18 (0-80)	0 (0-14)	901 (0-1575)

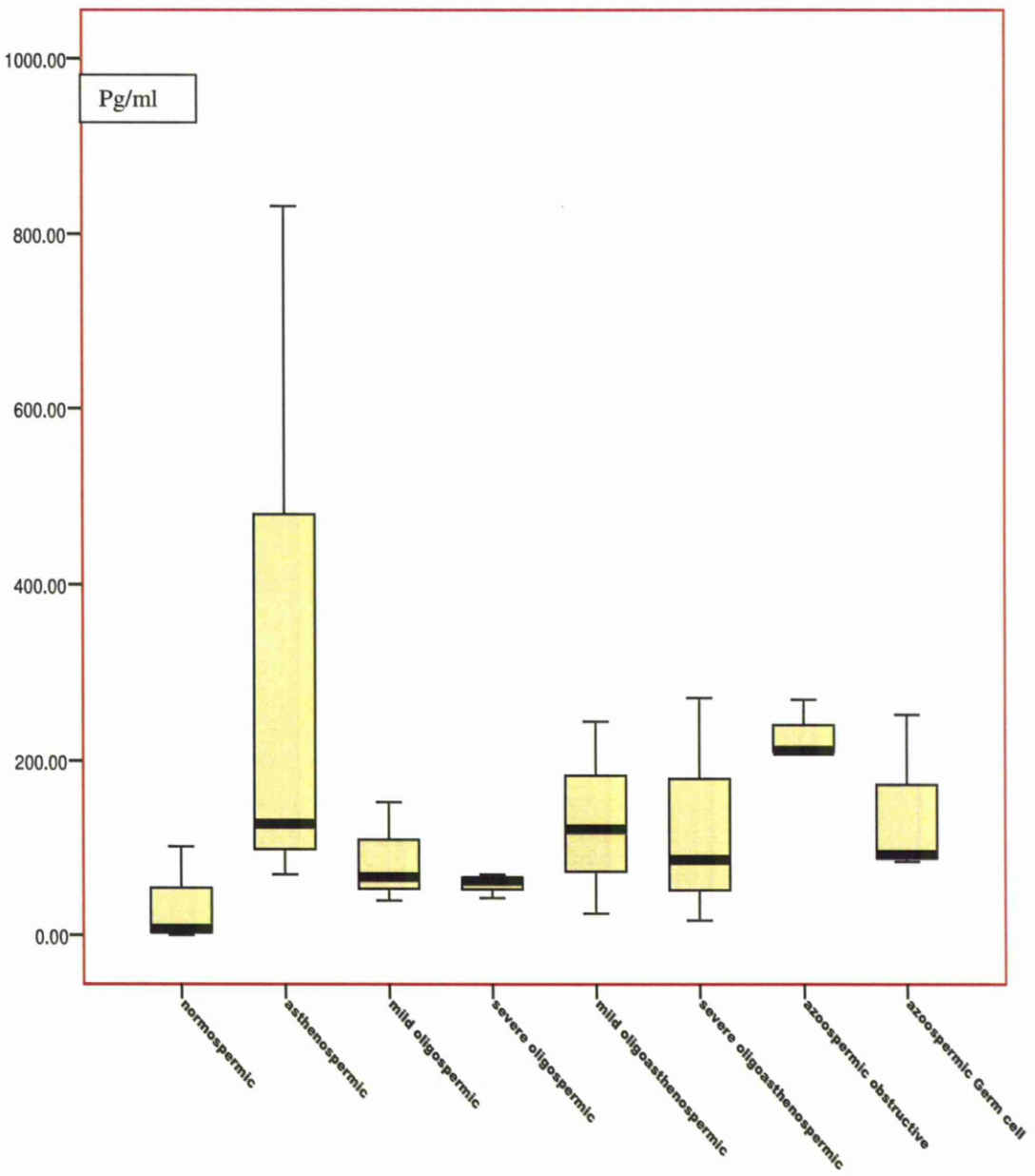


Figure 4.5: IL-10 levels (pg/ml) in the different subsets of subfertile men.

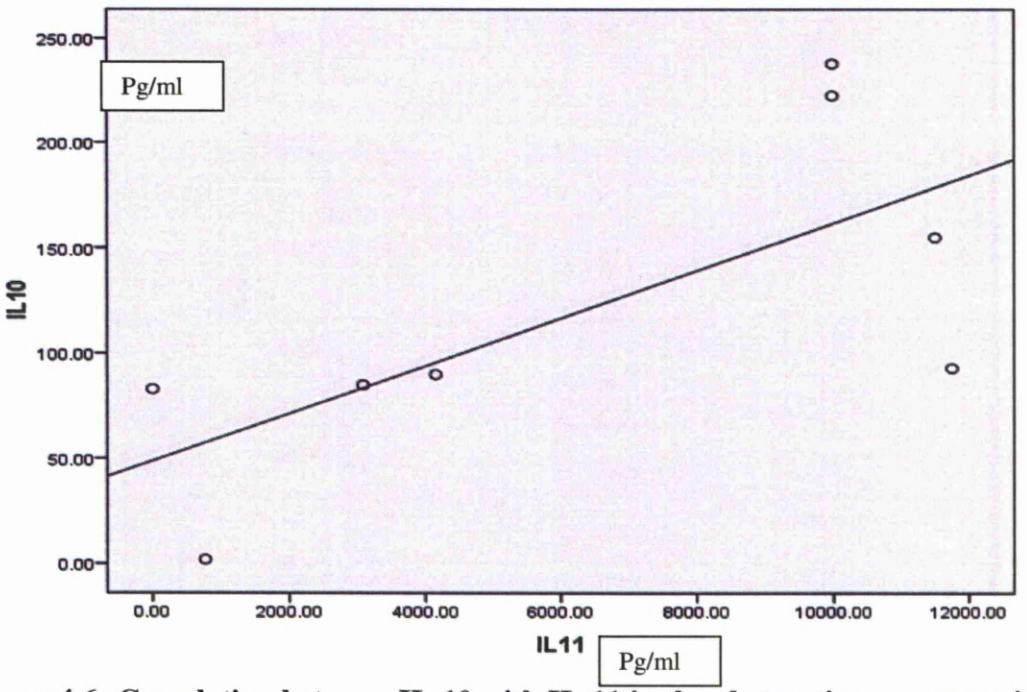


Figure 4.6: Correlation between IL-10 with IL-11 in the obstructive azoospermic group (r=0.75, p<0.01).

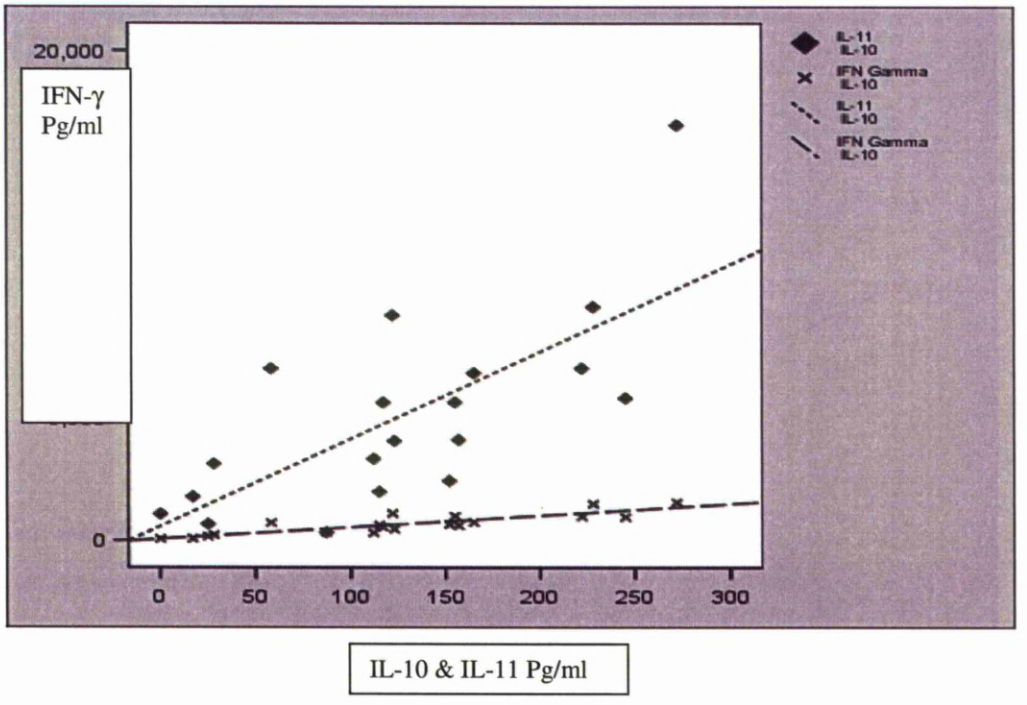


Figure 4.7: Correlation between IL-10 with IL-11 and IFN-γ pg/ml in the severe oligoasthenospermic group (IL-11 and IL-10 r=0.75, IFN-gamma and IL-10 r=0.84, p<0.01).

Furthermore, IL-6, IL-10, TNF-α and IFN-γ were significantly increased in the obstructive azoospermic group when compared to the normospermic group. A significant correlation was found between IL-10 and IL-11 in the oligospermic ($p<0.05$) and obstructive azoospermic ($p<0.01$) (Figure 4.6) groups. A significant correlation ($p<0.01$) was found between IL-10 and IL-11 and between IL-10 and IFN-γ in the severe oligoasthenospermic group as shown in Figure 4.7.

A significant correlation ($p<0.05$) was found between IL-10 and IL-6 in the severe oligoasthenospermic group. A significant correlation ($p<0.01$) was found between IL-10 and IFN-γ in the oligospermic and asthenospermic groups.

4.3.5 Discussion

IL-6 is produced by the Sertoli cells and has a number of effects including stimulation of transferrin production by Sertoli cells[128] and inhibition of meiotic DNA synthesis in pre-leptotene spermatocytes[129]. In this study, statistically significant raised levels of IL-6 were found in the mild oligospermic, severe oligospermic, asthenospermic and the severe oligoasthenospermic group. These results correlate with two previous studies where increased levels of IL-6 were found in the infertile groups[264;265]. Similarly, elevated levels of IL-6 have correlated significantly with sperm numbers in the ejaculate, sperm penetration rates in the sperm penetration assay, and some sperm motion parameters[254]. Increased levels of IL-6 were found in men with accessory gland infection when compared to normal men, but in contrast to this study, IL-6 levels did not show any significant differences between normal men and those with abnormal spermiograms.

In this study, there were significantly high levels of IL-6 in the obstructive azoospermic patients which would suggest that the testis may not contribute to the high IL-6 levels in the seminal plasma[171] and may be coming from the seminal vesicles and / or prostate. Similarly, significant correlations of IL-6 and fructose levels indicated that the seminal vesicles also take part in the production of seminal IL-6[266].

Increased amounts of IL-8 ($p<0.05$) were identified in the asthenospermic and obstructive azoospermic groups when compared to the normospermic group in this study. As IL-8 levels were increased in the obstructive azoospermic group this may suggest that the testis is probably not the site of production for this cytokine. The site of production for this cytokine could be inferred to be at the level of the epididymis by the fact that asthenospermic patients may have epididymal disease and the obstructed patients could have epididymal obstruction. The origin of IL-8 and its role in spermatogenesis and steroidogenesis has not yet been elucidated.

Statistically significant raised levels of IL-10 ($p<0.05$) were found in this study in the asthenospermic, severe oligoasthenospermic and obstructive azoospermic group when compared to the normospermic group. The latter suggest that the testis is probably not the site of its production. In contrast, high levels of IL-10 have been observed in the seminal plasma of fertile men when compared to infertile men with or without leucocytospermia[267]. The reason for this could be that in this work there was only a classification of infertile groups with or without leucocytospermia rather than individual analysis of cytokines in the different subgroups of infertile men.

This study found significant correlations between IL-10 and other proinflammatory cytokines in the oligospermic, asthenospermic, severe oligoasthenospermic and azoospermic groups. Raised levels of IL-10 were found along with raised levels of IL-6, IL-8 and TNF-α in the obstructive azoospermic group and raised levels of IL-10 were found along with raised levels of IL-6 and IL-8 in the asthenospermic group. IL-10 is an antiinflammatory cytokine with immunoregulatory functions and inhibits the production of several proinflammatory cytokines including IL-8 by altering the accessory cell function of macrophages[268]. Hence it is possible that the raised levels of IL-10 seen in this study were in direct response to suppress the proinflammatory cytokines such as IL6, IL-8 and IFN-γ. In contrast, IL-10 levels have been shown to be significantly decreased in the seminal plasma of oligo-teratospermia and asthenoteratospermic patients with genital infection when compared to fertile, azoospermic or oligo-terato-asthenospermic patients without infection[269].

Previously, increased levels of IL-11 were found in men with male accessory gland infection when compared to normal men, but IL-11 levels did not show any significant differences between normal men and those with abnormal spermiograms[262]. This study did not find any statistically significant difference in the levels of IL-11 amongst the various subgroups of subfertile men in comparison to normospermic group. This would suggest that this cytokine may not be influencing spermatogenesis.

This project demonstrated no significant difference in the IL-12 levels between the normospermic and subfertile subgroups. This is in agreement with other studies[269;270].

In this study, the TNF-α and IFN-γ levels were significantly increased ($p<0.05$) in the azoospermic obstructive group only. This would suggest that the testis is unlikely to be the

site of production of these cytokines. This study would support the finding of the study carried out by Eggert Kruse whereby TNF-α did not affect sperm numbers or motility. Furthermore, another study reported no differences in the TNF-α levels between fertile and infertile groups (bilateral testicular atrophy, male accessory gland infection, varicocele and post varicocelectomy: groups classified according to history, physical examination and sperm parameters) of men who attended a urology clinic[259]. In support of this study findings, there was no relationship between TNF-alpha levels in seminal plasma and semen quality or parameters of sperm functional capacity[252]. However, in this study the azoospermic men were excluded. Prominent cytokines associated with T cell function, such as IL-10, IL-12 and IFN-γ, were detected at a low concentration (< 100 pg/ml) in only a few samples of normal men. This is similar to the normospermic group values. This suggests that cellular immune activity is low in the genital tract of normal men[271].

However, in contrast to these findings, elevated levels of TNF-α and IFN-γ have been identified in the oligozoospermic as well as the azoospermic groups[272]. A study by Nabil et al. 2008 also showed significantly increased levels of TNF-α in the oligoasthenozoospermic and the azoospermic group when compared to the normospermic group. However, the azoospermic group was not subdivided into obstructive and non obstructive. TNF-α levels in seminal plasma have been shown to be negatively correlated with the number of progressively motile sperm in the ejaculate, but there was no correlation with total sperm counts, viability, morphology etc[177]. However, in a repertoire study of cytokines in seminal plasma, out of the cytokines tested (IL-1, IL-2, IL-4, IL-6, IL-8, TNF-α, IFN-γ, G-CSF, M-CSF andgranulocyte elastase) only IL-1, IL-8, TNF-α and G-CSF were significantly higher in the leucocytospermic group when compared to normospermic, azoospermic, oligospermic and asthenospermic groups[273].

IFN-γ exerts its inhibitory effects on testosterone production at the level of cholesterol transport into mitochondria[274]. IFN-γ is thought to stimulate the production of TNF-α and it is reported that there is a significant overproduction of IFN-γ levels in the seminal plasma of infertile men and a significant negative correlation with sperm count and motility[275]. However, in this study IFN-γ was elevated only in the obstructive azoospermic group.

As increased levels of cytokines in this study were found in the obstructive azoospermic group, the measured levels must be coming from either the seminal vesicles or the prostate. Although unlikely, there may even be a small contribution from the epididymis. The only definitive way to prove that the cytokines are not originating from the testis is to measure the cytokine levels from testicular sperm extraction samples.

This study confirms the effect of individual cytokines on sperm numbers and motility. It also confirms the hypothesis that there is an intricate cytokine network that exists in the seminal plasma as shown by the numerous correlations present in the various subfertile subgroups and their effect on sperm function. This cell mediated immunity could be up regulated by genital tract infection and lead to an increase in the levels of these cytokines[276].

***This study is published in the American Journal of Reproductive Immunology (Appendix 5).**

***Citation- Seshadri et al. The Role of Cytokine Expression in Different Subgroups of Subfertile Men. AJRI 2009; 62 (5): 275-282.**

4.4 STUDY NUMBER 4: Cytokine expression in IVF patients (Good fertilisers versus the Poor fertilisers)

4.4.1 Introduction

Cytokines are involved in gonadal and sperm function. Some *in vitro* studies have shown that cytokines such as IFN-γ and TNF-α decrease the motility of the spermatozoa[175;200]. Certain cytokines increase the generation of reactive oxygen species by human spermatozoa which increases the oxidative stress in the male genital tract[277]. Leucocytes and oxidative stress negatively affect the integrity of the sperm chromatin inducing DNA fragmentation[278] which increases morphological abnormalities of the spermatozoa[232]. Sperm DNA integrity impairment negatively affects embryo development[235].

It was hypothesised that there exists an intricate network of cytokines within the seminal plasma which may influence fertilisation rates either directly or indirectly. The aim of this study was to determine the function of the seminal cytokine network in relation to the fertilising capacity of sperm with regards to fertilisation rates and pregnancy outcomes in a subsequent IVF cycle. In most studies, only a few cytokines (up to four) in seminal plasma have been measured, and the pathophysiological effect of these cytokines on fertilisation rates and pregnancy outcomes has remained largely unexplored.

In this study the cytokines were measured in the ejaculate at the time of IVF so it represented the true physiological situation since those sperm were used to inseminate and thereafter fertilise the oocytes. To our knowledge this is the first study testing seven cytokines in the seminal plasma of men attending IVF and comparing cytokine levels with fertilisation rates and pregnancy outcomes in an IVF cycle. These cytokines were selected as they have been

studied previously either individually or as pairs with conflicting results. The hypothesis was also tested by finding out if there was any correlation amongst the cytokines to thus determine whether cytokines act individually or more as a network, which could be one reason for the conflicting results of previous studies.

4.4.2 Materials and Methods

Study Design

As outlined in Chapter 2 section 2.6.2. Semen was obtained from the partners of women attending for IVF cycle, by masturbation after three-five days of sexual abstinence. The samples were collected and analysed similar to group 1 as described in section 2.2. Couples undergoing traditional IVF only were chosen so as to study the effect of the various cytokines on IVF outcome rather than sperm parameters. The inclusion criteria for the men were that they neither had illnesses nor did they take any medication that could have affected cytokine metabolism. These men did not smoke or drink on a regular basis. These men formed the control group.

Different grading schemes are in use to grade embryos when they reach the blastocyst stage (around day three of their development). An in house grading system of Grades 1-4 as outlined in Chapter 2 section 2.3 (Figure 2.1) was used. Although grading systems vary slightly from one unit to another, they all produce similar results.

4.4.3 Statistical Analysis

The analysis was performed as outlined in Chapter 2 section 2.6.3.

4.4.4 Results

36 men were recruited into this study. The results of the seminal analysis from all patients are shown in Table 4.6. Sperm motility ranged from 20% - 100%. There was no correlation between sperm morphology and count when compared with the fertilisation rates. The embryos were graded from 1 to 4. There were 28 Grade 1, three Grade 2 and one Grade 3 embryos. The age range of the female partners was 23-42 years with a median of 34 years. The causes of their primary infertility included tubal factor (n=18), ovulatory (n=1) and unexplained (n=10).

Cytokine levels in the seminal plasma

IL-6, IL-8, IL-10, IL-11, were detected in all samples and IL-12, TNF-α and IFN-γ were detected in most samples. It was not possible to assay all cytokines in all 36 samples as some of the volumes of the ejaculate were limited. Due to insufficient specimen samples it was not possible to test some cytokines such IL-8, IL-11 and TNF-α in all the samples. There was no conscious decision made to test some cytokines more than the others. However, this is one of the limitations of this study as the correlation between ejaculate volume and cytokine levels could have been overlooked. Table 4.7 shows the median and ranges for all cytokines. IL-8 and IL-11 were present at higher levels than other cytokines.

The men were classified according to the fertilisation rates achieved by IVF. Four samples were excluded from the analysis as there was no data on the fertilisation rates. However, there was no correlation between cytokine concentration and fertilisation rates for any cytokine. Further analysis was performed by dividing men into good fertilisers (men who had a fertilisation rate above 60%) and poor fertilisers (fertilisation rate below 35%).

Table 4.6: Clinical data and sperm parameters of the men attending IVF.

Parameters	Median (Range)
Age (in years)	34 (25-45)
Sperm motility (%)	90 (20-100)
Normal morphology (%)	38% (9-67%)
Sperm count (x 10^6/ml)	90 (38-186)
Round cells (per HPF) HPF- High Power Field	2 (0-5)
Fertilisation rate (%)	60 (0-100)
Cleavage rate (%)	60 (0-100)
Biochemical pregnancies	12/32
Clinical pregnancies	10/32

Table 4.7: Median and the inter-quartile ranges of the different cytokines in pg/ml.

Cytokines pg/ml	N	Median (pg/ml)	Inter quartile range (pg/ml)
IL-6	36	36	24.5 - 71.5
IL-8	31	1150	380 - 1800
IL-10	36	70.25	34.17 - 100.42
IL-11	34	3575	2675 - 5475
IL-12	36	11	5 - 15
TNF- α	30	9	0 - 18
IFN-γ	36	79	46 - 318

The arbitrary values of 60% and 35% were set as a result of a decision made by the IVF unit as 60% is the average fertilisation rate for the unit. It was thought that if a significant difference were to be found between the poor fertiliser and good fertiliser group, then further analysis would be carried out by looking at the men with fertilisation rates between 35%-60%. Furthermore, this would also give an insight into the role of cytokines in the extremes of fertilisation rates. The remaining men who had fertilisation rates between 35% and 60% (n=6) were excluded from analysis. The cytokine levels of the poor and good fertilisers groups are shown in Table 4.8. Levels of IL-11 were significantly higher in the good fertilisers group (Mann-Whitney U=39.5; p=0.05) (Figure 4.8).

Correlation between cytokines

Spearman's non-parametric test was used to measure the correlations between cytokines. A positive correlation was found between IL-8 and IL-6 (∞ 0.40, $p<0.03$) (Figure 4.9). A positive correlation also exists between IFN-γ with IL-10 and IL-11 (∞ 0.36, $p<0.04$ and ∞ 0.69, $p<0.0001$) respectively (Figure 4.10). There was also a positive correlation between IL-10 and IL-11(∞ 0.53, $p<0.001$).

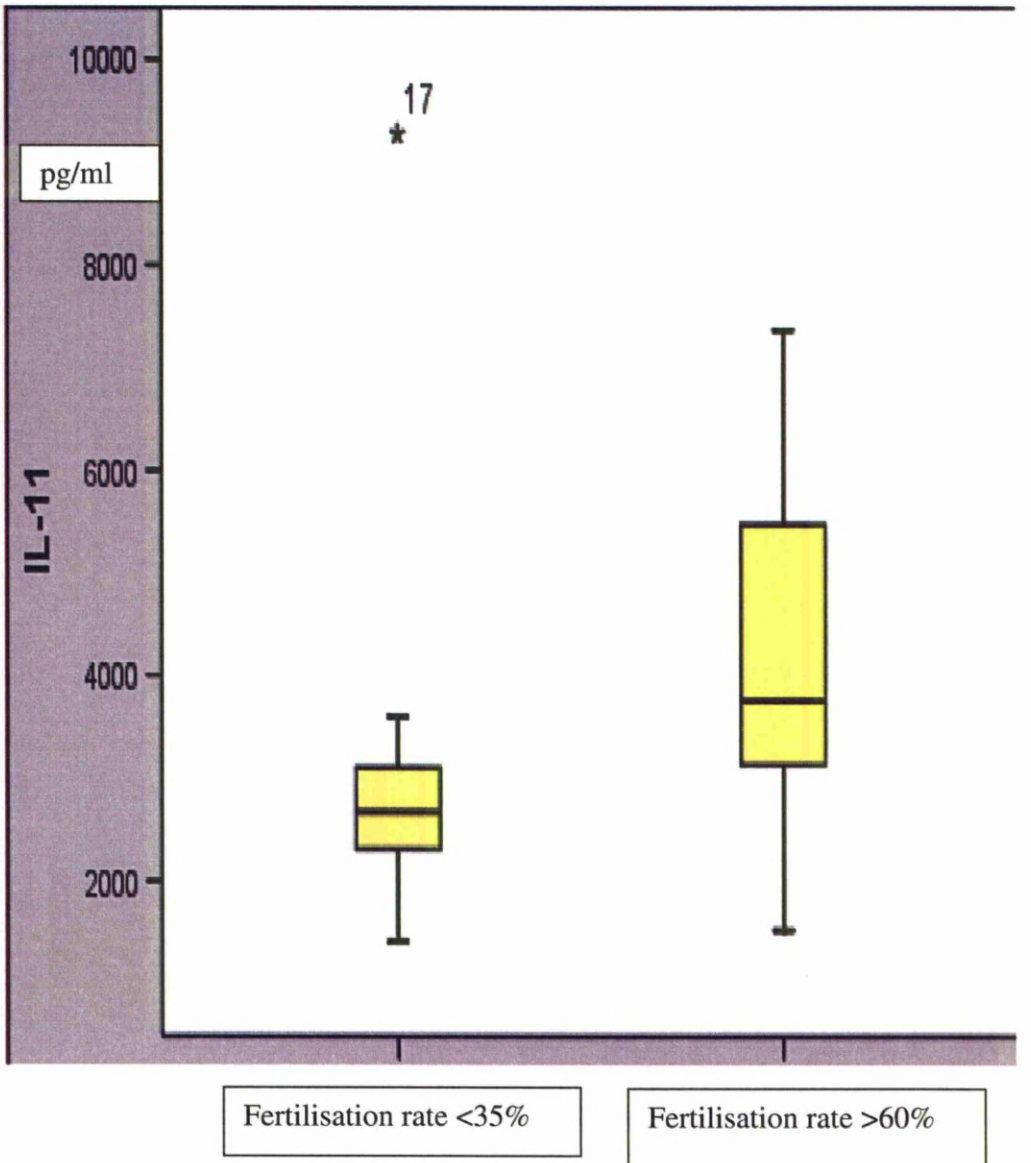


Figure 4.8: Significant levels of IL-11(pg/ml) in group.

Table 4.8: Cytokine levels (pg/ml) in men with fertilisation rates < 35% & > 60%.

Cytokines pg/ml	Fertilisation rate < 35%			Fertilisation rate >= 60%		
	N	Median (pg/ml)	Inter quartile range (pg/ml)	N	Median (pg/ml)	Inter quartile range (pg/ml)
IL-6	9	26	23 - 65	17	35	29 - 49
IL-8	7	900	450 - 1400	14	1425	450 - 1800
IL-10	9	40.83	25 - 100.83	17	68.00	30.83 - 99.17
IL-11	9	2675	2317 - 3100	17	3750	3133 - 5475
IL-12	9	11	7 - 14	17	11	0 - 15
TNF- α	6	7.5	0 - 17	14	10	0 - 19
IFN-γ	9	57	47 - 72	17	68	35 - 212

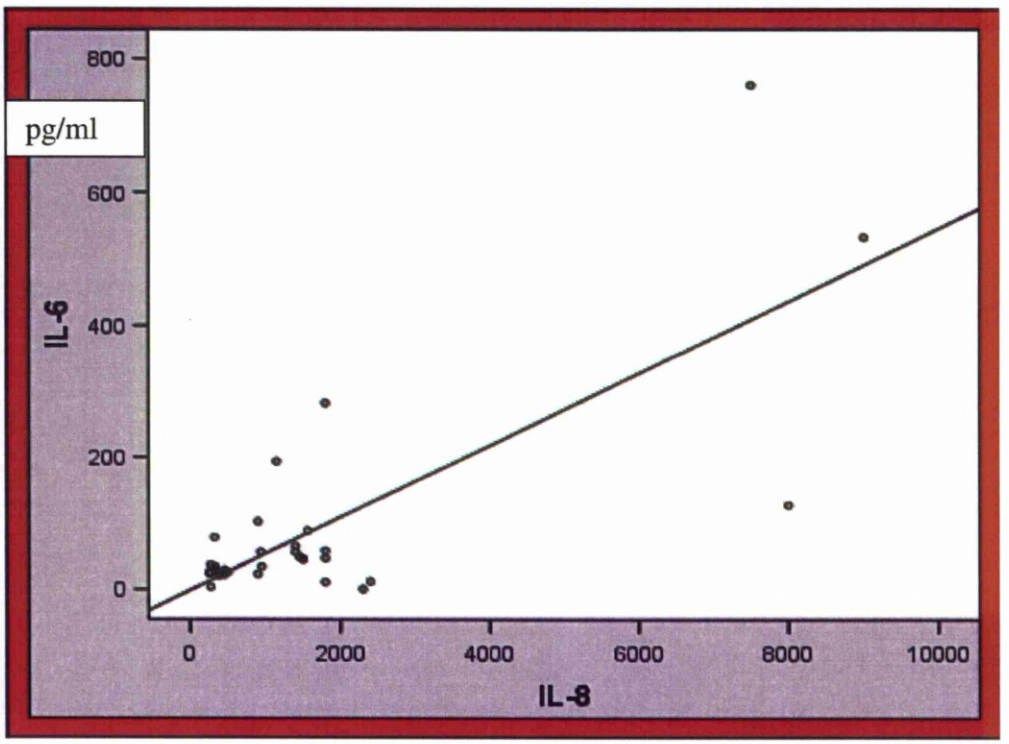


Figure 4.9: Positive correlation between IL-6 and IL-8 levels (pg/ml).

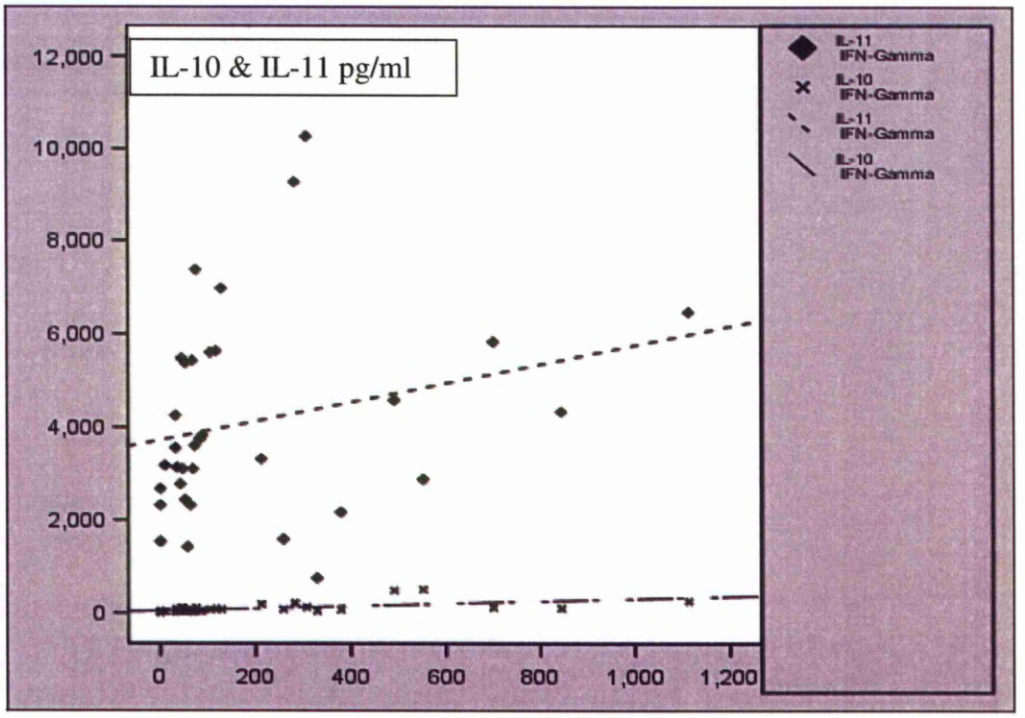


Fig 4.10: Correlation of IFN-gamma with IL-10 & IL-11 (pg/ml).

4.4.5 Discussion

The IL-6 level found in this study was slightly higher by about 4 (pg/ml) than in previous published studies[177;279&280]. The effect of IL-6 on sperm is unclear[281]. Some studies have failed to demonstrate any effects of IL-6 on the semen although others have[171;259;266]. One study demonstrated an inverse correlation between sperm number and motility parameters and seminal IL-6 levels[282]. IL-6 has been found to enhance the fertilising capacity of human sperm cells *in vitro* in some studies[179] but not others[283;284]. In this study there was no correlation between IL-6 levels and fertilisation rates. This is in agreement with other studies showing IL-6 levels are not associated with the outcome of the sperm cervical mucus penetration test[251;285].

In this study, although the levels of IL-8 were higher in the good fertilisers group, they did not reach statistical significance. There was no correlation between IL-8 levels and fertilisation rates in this study. This is in agreement with another study, in which there was no reported correlation between IL-8 and sperm parameters[286]. Conversely, IL-8 has been reported to exert a negative effect on the fertilising potential of spermatozoa[277].

IL-10 was detected in the seminal plasma of normal men in this study and was found to have no effect on the fertilising capacity. These data are similar to other studies[267;269]. IL-10 is an antiinflammatory cytokine and is produced mainly by macrophages and T cells and suppresses the production of various proinflammatory cytokines[287]. High levels of IL-10 have been observed in the seminal plasma of fertile men[267]. Significantly decreased levels of IL-10 have been found in the seminal plasma of infertile men[269].

There have been only a few studies analysing the levels of IL-11 in seminal plasma. In this study, IL-11 levels detected were considerably higher than other studies[262]. Significantly higher levels of IL-11 were found in the good fertilisers group. In one previous study no correlation was found between IL-11 levels and variables of the spermiogram[262]. Another study showed that significantly higher levels of IL-11 were present in the seminal plasma of infertile men with genital infection and oligo-terato-asthenospermia[140]. The role of IL-11 in seminal plasma and its relationship to the fertilising capacity of sperm has not been fully evaluated. Further studies need to determine the role of IL-11 in seminal plasma and in preimplantation embryo development.

This study detected IL-12 in the seminal plasma; however, there was no correlation between the levels of IL-12 and the fertilisation rates. The levels of IL-12 detected in normal men have differed between various studies[253;269;270]. IL-12 is an immunoregulatory polypeptide growth factor and is produced by a wide variety of cells including macrophages, neutrophils and is known to promote both the growth of natural killer cells and cytotoxic T cell activity. It also induces the secretion of interferon-γ and promotes the growth of activated T and NK cells[288-290]. Leucocyte free infertile men have been seen to have significantly lower levels of IL-12 compared to fertile men, indicating a direct or indirect role for leucocytes in male fertility[253]. The exact pathophysiological pathway involved in the modulation of IL-12 levels as related to the reproductive function of the sperm requires further investigation.

The levels of TNF-α detected in this study mirror the levels in other studies[177;259]. This study showed no relation between the levels of TNF-α and fertilisation rates. TNF-α has adverse effects on sperm motility[291;259]. Results of a different study showed the levels of TNF-α and IFN-γ are elevated in human semen during inflammation related infertility[292].

The results of another study showed that when spermatozoa are exposed to TNF-α and IFN-γ in combination, the viability and integrity of the spermatozoa are compromised thereby indirectly contributing to the fertilising potential of the sperm[293]. It is possible that only at high concentrations TNF-α affects the fertilising capacity of the sperm. However, further studies are needed to confirm this hypothesis.

This study was able to detect IFN-γ in all the seminal plasma samples collected. This is in agreement with the data presented by others[275]. This study showed no relation between IFN-γ and fertilisation rates. IFN-γ, a secretory product of activated T cells and natural killer cells has been hypothesised to have a toxic effect on sperm function. IFN-γ has been demonstrated *in vitro* to adversely affect sperm motion parameters[200] and the fertilising ability of human sperm to penetrate zona-free hamster eggs[163]. *In vivo* data on IFN-γ concentrations in a relatively small number of seminal plasma samples have been published, showing no significant difference in IFN-γ levels between fertile and infertile subjects. However, in another study, increased IFN-γ concentrations in the seminal plasma of infertile subjects was found[275]. Further studies are required to assess the role of IFN-γ and TNF-α in seminal plasma.

There are a few limitations to this study. The numbers of patients in each group are small as this was meant to be a pilot study. Hence statistical significance may not correlate with clinical significance. A revised definition on sperm quality has been published by WHO in 2010. However, it is unlikley that the 2010 definition would have changed much of these results or interpretations. Although it has been endeavoured to classify the patient groups as good and poor fertilisers using an arbitrary point of 60% and 35% respectively, some conventional authors may not agree with the quoted reference ranges of semen parameters. They could have preferred to have even compared the upper quartile to the lower quartile.

The relevance of this study could have been increased by the measurement of the same cytokines in the oocyte / embryo culture medium. It would have also been useful to look at the number of biochemical / clinical pregnancies resulting from the embryos created by the good and poor fertilisers. This would have then led to further discussion about the correlation / relationship between pregnancy outcome and the seminal plasma cytokine content.

This study has shown that six cytokines do not affect sperm-oocyte interaction and fertilisation rates in IVF. However, it appears that IL-11 could have a role in the fertilising capacity of the sperm. Significant correlations between cytokines have also been identified. The modulation of this cytokine network could explain the wide variability of the success rates seen in IVF when male factor is ruled out by normal semen analysis. The sperm is washed and prepared prior to IVF. The aim of washing and preparation of the sperm are to separate sperm from the seminal plasma which contains the cytokines. The process of sperm preparation during IVF thus removes any potential adverse effect of cytokines on sperm capacitation and sperm-oocyte interactions.

"Cytokine expression in the seminal plasma and its effects on fertilisation rates in an IVF cycle". *This study has been accepted for publication in the Andrologia Journal.

CHAPTER 5: LEUCOCYTES AND CYTOKINES IN SUBFERTILE MEN

5.1.1 Introduction

Little is known about the physiological significance of the inflammatory cells and cytokines in seminal plasma in terms of their effects on uterine receptivity to embryo implantation and pregnancy. There is gathering evidence that human seminal plasma lymphocytes and cytokines influence implantation. Hence, there is a necessity to understand the role of lymphocytes and cytokines in seminal plasma.

Evidence is accumulating to support the hypothesis that insemination is causally linked to the activation and expansion of populations of lymphocytes mediating forms of active immune tolerance in the implantation site[294]. Several cytokines activated by semen are attributed with regulating proliferation, viability and differentiation of blastomeres in the embryos[295;296]. Cytokines targeting the developing blastocyst include IL-6 and leucocyte inhibitory factor that are induced after exposure to semen[297;298].

There is some evidence from the previous two Chapters (3 and 4) to show that neither leucocytes nor cytokines on their own affect male fertility significantly. However, in combination they could lead to male subfertility. The hypothesis was to confirm the presence of cell mediated response in the seminal plasma and the main aim of this study was to identify any correlations between the different leucocyte subpopulations and the individual cytokines and their combined effect on sperm parameters.

5.1.2 Materials and Methods

Immunohistological techniques as outlined in Chapter 2 sections 2.5.1, 2.5.2, 2.5.3 for leucocytes and cytokine ELISA sandwich method as outlined 2.6.2 for cytokines was used.

5.1.3 Statistical Analysis

The analysis was performed as outlined in Chapter 2 sections 2.5.5 for leucocytes and 2.6.3 for cytokines.

5.1.4 Results

The results were drawn from the same group of patients in whom the leucocytes and cytokines were studied independently in Chapters 3 and 4 respectively. Further analysis was performed to find out if any significant correlation existed between the leucocytes and cytokines in various groups of sub-fertile men as described in the previous chapters. Significant positive correlations were identified between the T cells and B cells and the various cytokines in the different subfertile groups. IL-6 a proinflammatory cytokine positively correlated with the T cell (CD3), B cell (CD20) and MHC class II (L243) in the ASA group as shown in the correlation matrix in Figure 5.1 (IL-6, L243, CD20 with CD3 $r=0.67$, IL-6 and L243 $r=0.69$ p-value<0.01). Similarly IL-6 also correlated positively with activated T and B cells (CD69) and IL-8 positively correlated with macrophages and monocytes (CD14) in the culture positive group as shown in Figure 5.2 (IL-6 and CD69 $r=0.56$, IL8 and CD14 $r=0.67$, $P<0.01$). This finding correlates with previous studies which have noted increased levels of both IL-6 and IL-8 in men with accessory gland infections[273;286]. IL-6 can cause sperm damage through the activation of activated T and B cells (CD69) and IL-6 was significantly elevated in the oligospermic, asthenospermic and azoospermic groups whereas IL-8 was elevated in the asthenospermic and azoospermic group.

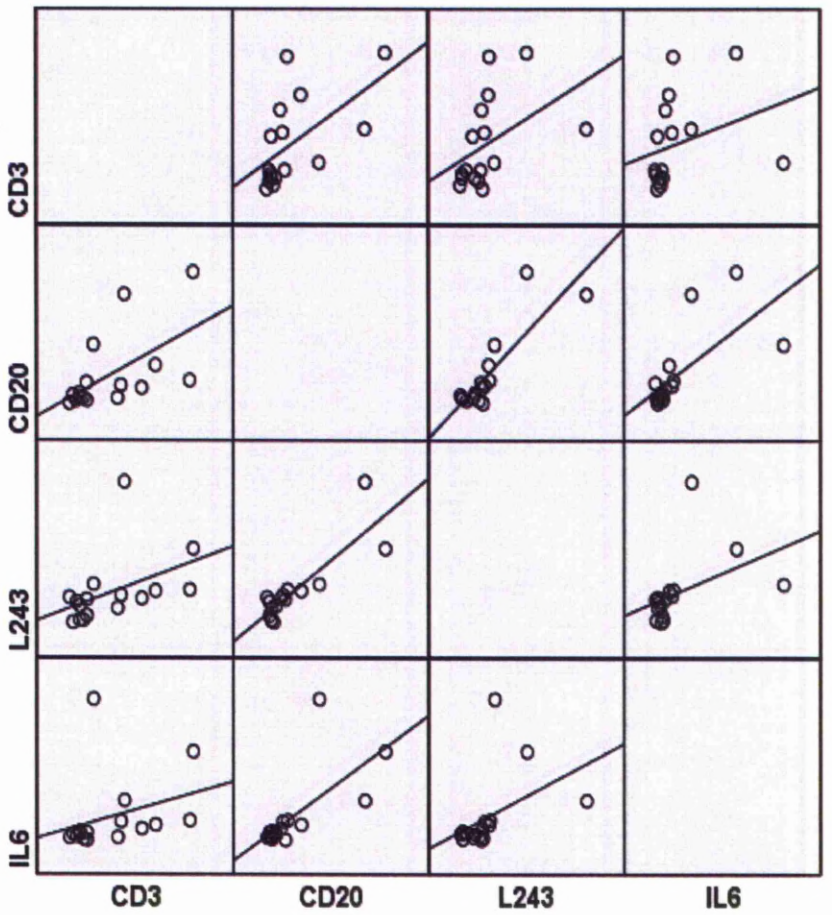


Figure 5.1: Correlation matrix depicting the correlation between T and B cells (expressed as cells per 10 hpf) with IL-6 (pg/ml) (IL-6, L243, CD20 with CD3 r=0.67, IL-6 and L243 r=0.69 p value < 0.01) in the ASA group.

A positive correlation was detected between IL-11, IFN-γ and monocytes and macrophages (CD14) in the general culture positive group as shown in Figure 5.3. Although IL-11 has been found in men with male accessory gland infection, it hasn't been found to affect spermatogenesis in this study. Although specific association of IFN-γ and CD14 has never been studied before, it could lead to sperm membrane lipid peroxidation which could be important in the sperm fecundation process.

IL-6 positively correlated with L243 in the mild oligospermic group as shown in Figure 5.4 ($r=0.83$, p-value<0.01) and IFN-γ negatively correlated with CD20 in the severe oligospermic group as shown in Figure 5.5 ($r=-0.74$, $p<0.05$).

IL-10 positively correlated with CD69 in the asthenospermic group as shown in Figure 5.6 and IFN-γ positively correlated with CD69 and L243 in the same group as shown in Figure 5.7. IFN-γ correlation between the various leucocyte subpopulations in different subferile men could suggest that IFN-γ could lead to increased sperm membrane lipid peroxidation.

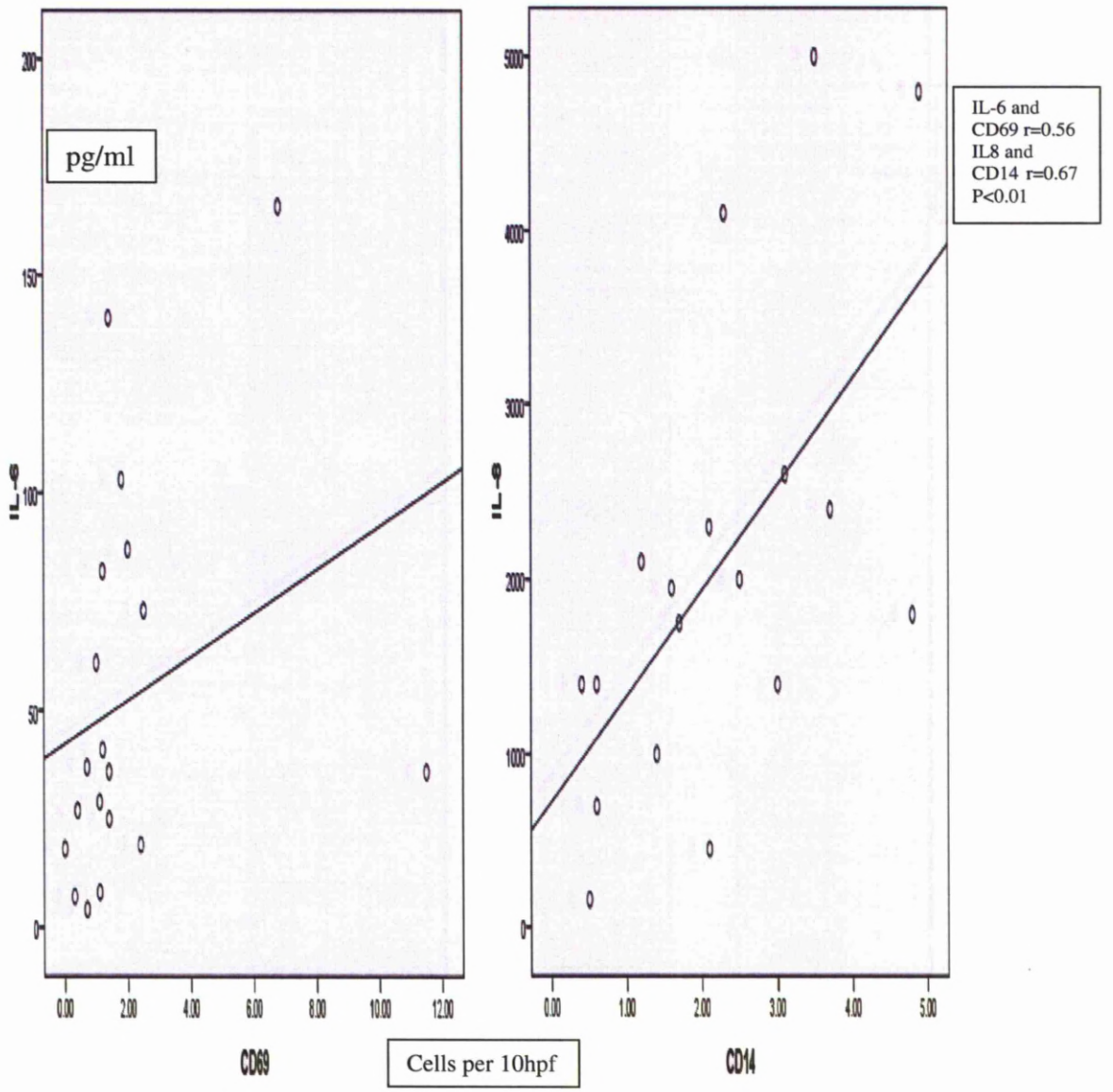


Figure 5.2: Correlation between IL-6 and IL-8 (pg/ml) and CD69 (activated T and B cells) and CD14 (expressed as cells per 10 hpf) respectively in the culture positive group.

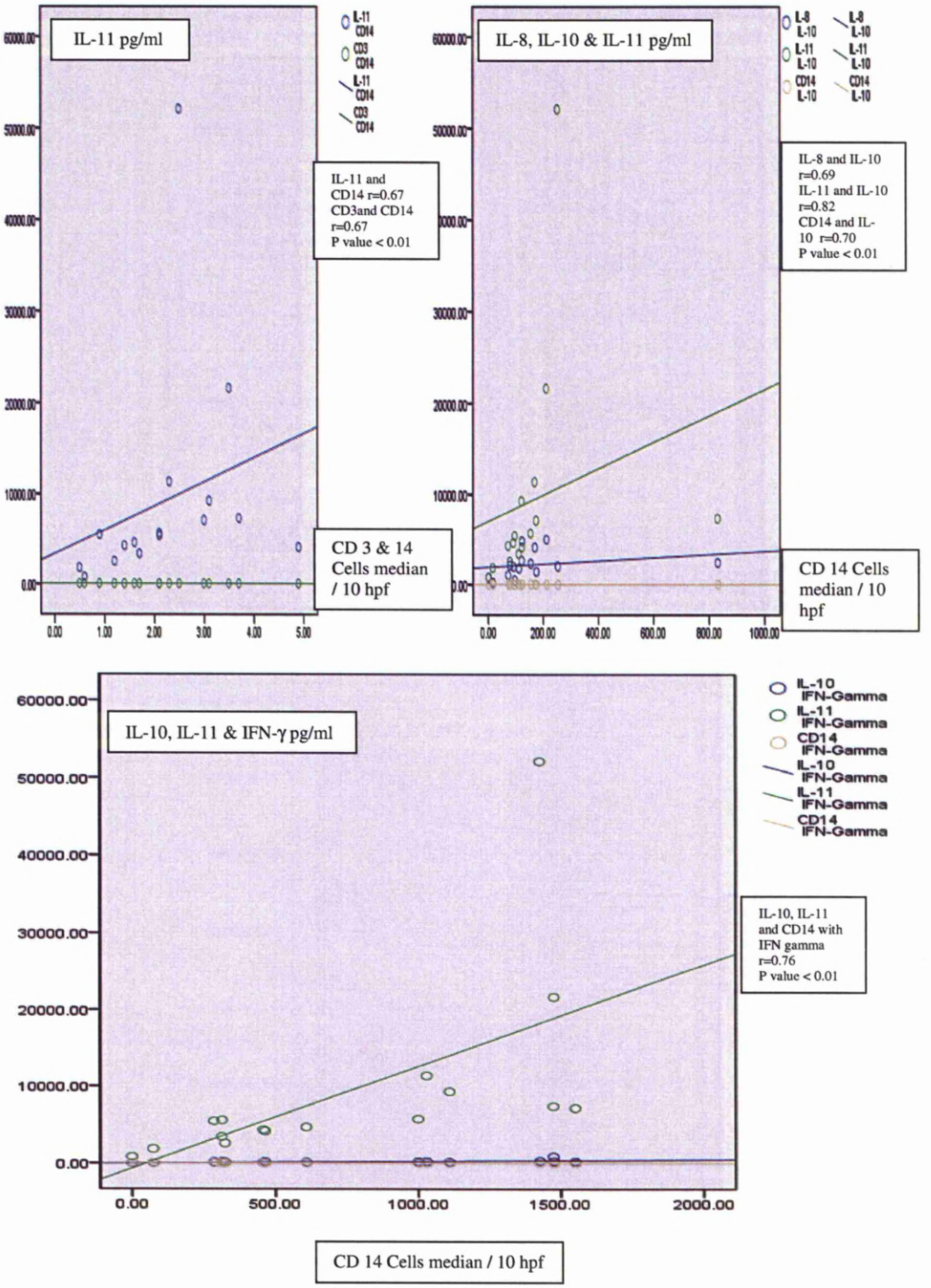


Figure 5.3: Correlations between the leucocytes (expressed as cells per 10 hpf) and cytokines (pg/ml) in the general culture positive group.

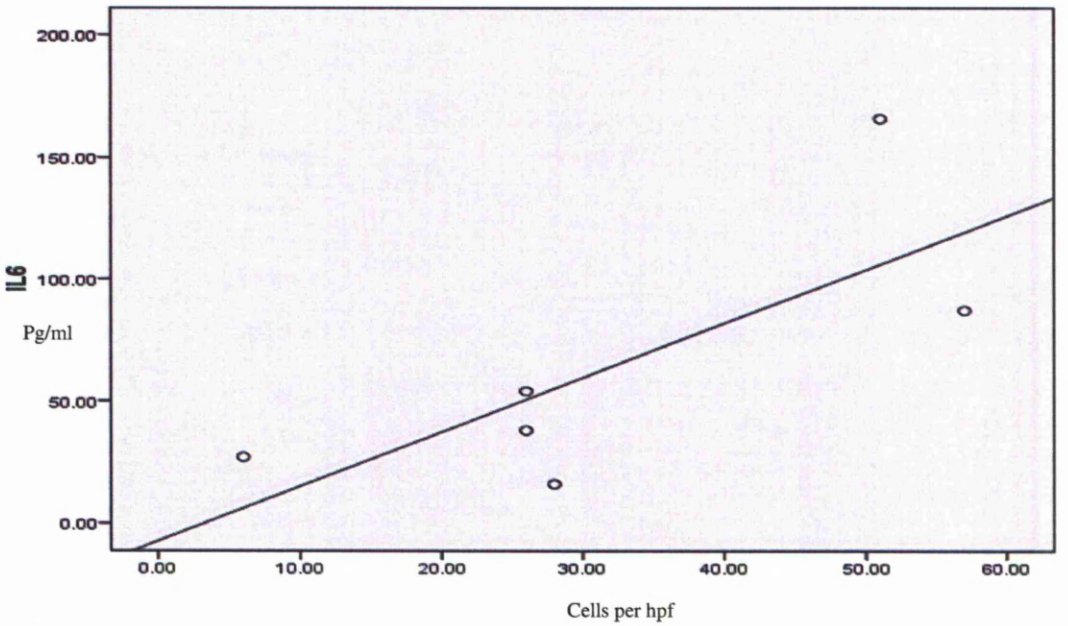


Figure 5.4: Correlation between leucocytes and cytokines in the mild oligospermic group (r=0.83, p value< 0.01).

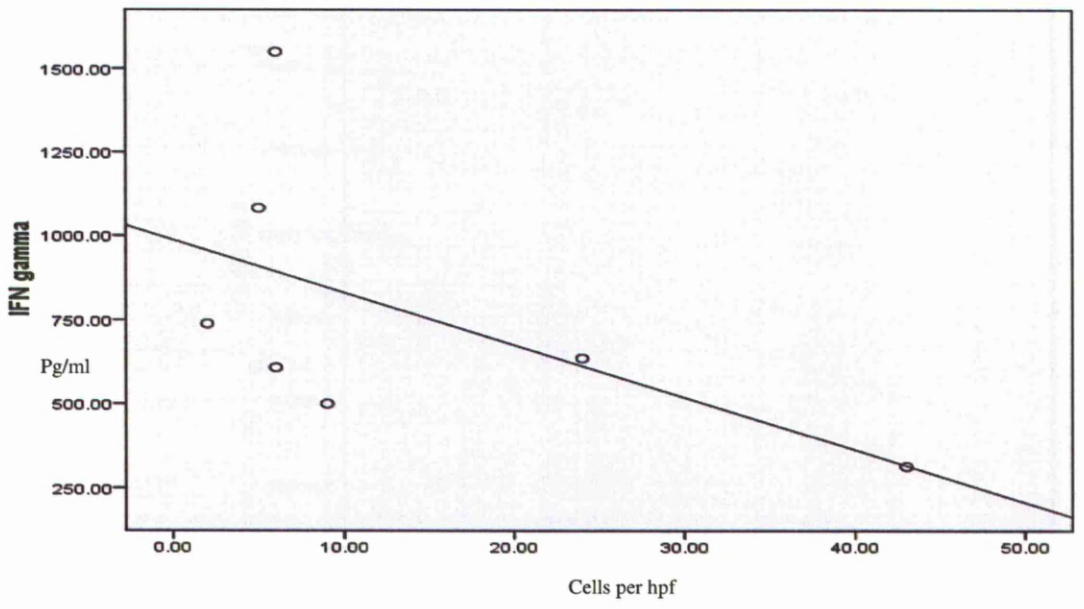


Figure 5.5: A negative correlation between CD20 and IFN-gamma in the severe oligospermic group (r=-0.74, p<0.05).

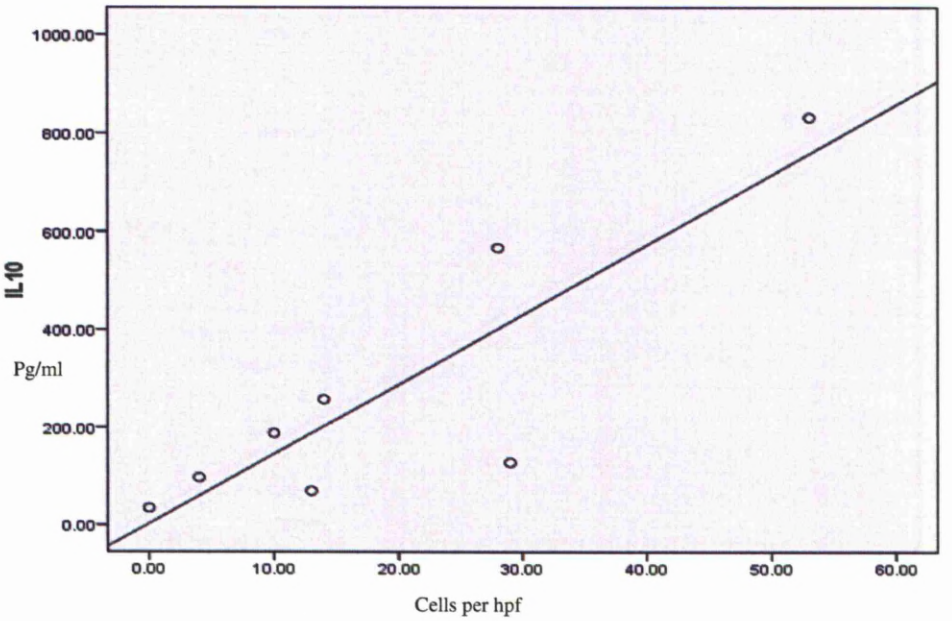


Figure 5.6: Correlation between leucocytes and cytokines in the asthenospermic group (r=0.88, p<0.01).

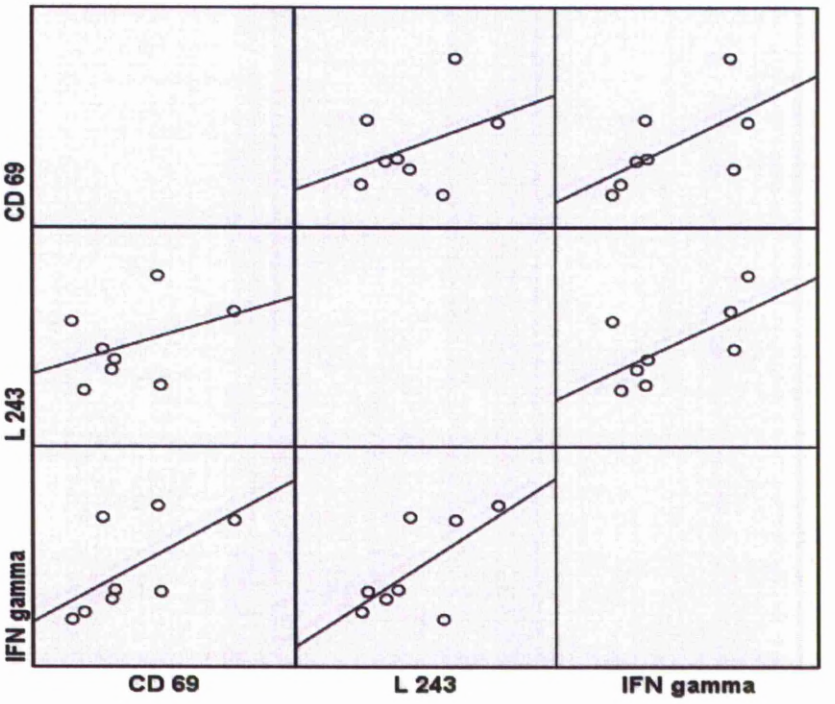


Figure 5.7: Correlation matrix between cytokines (pg/ml) and leucocytes (expressed as cells per 10 hpf) in the asthenospermic group (r=0.86, p value < 0.01).

5.1.5 Discussion

In this study, in the ASA positive group, IL-6 positively correlated with the presence of T and B cells. It is possible that CD3 positive T cells could induce CD20 positive B cells to release both IL- 6 and antibodies. IL-6 expression correlated positively with activated number of T and B cells and IL-8 positively correlated with macrophages and monocytes (CD14) in the culture positive group as shown.

CD14 mediates the induction of IL-8 and this leads to inflammation. Although there is only limited evidence for this in the seminal plasma, this is thought to be the process involved in other inflammatory pathology including periodontal pathogens[299] and in *Helicobacter pylori* (*H. pylori*) infection[300]. IL-8 increased the levels of sperm membrane lipid peroxidation at physiological concentrations and also showed a higher effect when assayed in the presence of leucocytes, at infection-inflammation concentrations. Thus, IL-8, either alone or in the presence of leucocytes, could drive the lipid peroxidation of the spermatozoa plasma membrane to levels that can affect the sperm fertility capacity[301].

No significant correlations were detected between the various leucocyte subpopulations and the cytokines IL-10, IL-11 and IL-12 in the various subfertile group studied. This would strongly suggest that these cytokines do not influence spermatogenesis. However, IL-11 may have a role in male reproductive capacity as this was elevated in the good fertiliser group. IL-11 is expressed in the developing spermatogonia in the testis and is developmentally regulated. It is also been shown to play a role in the recovery of spermatogenesis following cytotoxic therapy[302]. It has been shown that lack of IL-11 receptors leads to defective decidual response to implantation[303].

No correlations were evident between TNF-α expression and the leucocyte subpopulations studied. However, another study found TNF-α level in the leucocytospermic group to be significantly higher than those in the normal and asthenospermic groups. The reason for the difference observed between this study and the above study could be due to the fact that the patients in the current study probably had sub-clinical infection and thus were not mounting a significant immune response. Furthermore, none of the patients in this study tested positive for Chlamydial infection.

Similarly to the case with IL-8, it has been shown that physiological levels of TNF-α can increase the levels of sperm membrane lipid peroxidation and also showed a higher effect when assayed in the presence of leucocytes. Thus, TNF-α, in the presence of leucocytes, could drive the lipid peroxidation of the spermatozoa plasma membrane to levels that can affect the sperm fertility capacity[304].

The biological role and the clinical significance of increased numbers of leucocytes in semen remains a subject of controversy. The determination of leucocyte counts in semen is important but not critical for the detection of infection or inflammation in the male reproductive tract[304]. Moreover, rather than the leucocyte numbers in semen, their activity levels decide the final effects of oxidative stress on spermatozoa[170]. In addition, the presence of activated leucocytes in semen after elimination of an infectious agent may delay recovery of the normal oxidative balance in semen[170]. Cytokines participate in signal transmission between cells and perform regulatory roles in different biological processes, such as cell activation, proliferation, growth, differentiation, and mobility. They also show modulatory effects on inflammatory reactions.

The course of the inflammatory reaction depends on the levels of cytokines produced, as well as the presence of cytokine inhibitors and their specific receptors and / or antagonists. When they occur together, they can act synergistically, additively or antagonistically on the function of the target cell.

Many authors have observed correlations between the levels of proinflammatory cytokines and the number of leucocytes in semen[305;306]. However, there have also been reports demonstrating the elevated levels of proinflammatory cytokines in semen, regardless of the presence or absence of leucocytes[307]. Leucocytes mediate the induction of ROS generation by proinflammatory cytokines. Furthermore, oxidative stress that appears in leucocytospermia is possibly exerted by the increased levels of the cytokines themselves. ROS (generated by leucocytes) acts synergistically with proinflammatory cytokines to exacerbate the destructive environment for the spermatozoa.

Several proinflammatory cytokines at physiological concentrations increase the level of lipid peroxidation of sperm membranes, which could be important for the sperm fecundation process. However, as mentioned above, increased concentrations of some cytokines such as IL-8 and TNF-α either alone or in the presence of leucocytes, could drive the lipid peroxidation of the spermatozoa plasma membrane to levels that can affect the sperm fertility capacity[304].

A causal relationship between either leucocytospermia individually or cytokines on their own and subnormal sperm parameters (numbers, morphology, motility) could not be conclusively established in this study. However, what this study has shown is that there is a fine balance in the seminal plasma with multiple correlations amongst the various T cells, B cells, cytokines

and antioxidants which help in maintaining male fertility. When this fine balance is tipped (i.e. infections, ASA, obstruction etc.) new correlations appear amongst the different populations of leucocytes and cytokines which are detrimental to sperm and may result in infertility.

Correlations between the cytokines and leucocytes observed on the various subfertile men in this study confirm that synergistically they damage the spermatozoa, most likely by intensifying the sperm cells membrane peroxidation, affecting their redox profile as well as their close environment. Thus, the harmful effect of cytokines on spermatozoa is closely connected to the accompanying leucocytospermia. Although the evaluation of leucocyte concentration in semen still remains important, it is on its own insufficient in the diagnosis and treatment of male genital tract infection / inflammation. The assessment of sperm DNA integrity could provide further useful information in explaining the pathologic role of leucocytes and cytokines in male subfertility.

Conclusion

The results of this study indicate that there is a marked relationship between some proinflammatory cytokines and leucocytes which affects semen quality.

CHAPTER 6: CONCLUSIONS

6.1.1 Introduction

The predominance of T lymphocytes in the seminal plasma detected in this work would explain the significant amounts of various cytokines detected in the different subfertile groups in our study. Despite new techniques in the detection of the various seminal cytokines, the pathophysiological significance of the cytokines in sperm function is still unclear and controversial.

Cytokines rarely act in isolation, but rather act to induce or inhibit other cytokines, creating a population or network of cytokines to which cells respond[308]. Elevated levels of IL-6, IL-8 and IL-11 have been found in the seminal plasma of infertile men with infection and oligo-terato-asthenozoospermia[140]. Hence, we wanted to see if there was a significant correlation between the different cytokines in subfertile men. It was thought that this cytokine network would render individuals more or less resistant to particular infections[258].

To the best of our knowledge, no previous studies have concurrently analysed the simultaneous expression of both leucocytes and seven cytokines in the seminal plasma of men belonging to different groups of male subfertility. The role of cytokines in the fertilising capacity of sperm has not been fully evaluated either.

6.1.2 Results summary

Leucocytes

A low CD4/CD8 ratio might be of diagnostic value in detecting ASA, as the ratio of T helper cells to cytotoxic T cells in the ASA + group is 1.37 in comparison to the control group which was 2 (Section 3.1).

There was no statistical significant difference between the culture (ureaplasma and other general culture organisms) positive and negative groups with regard to sperm parameters or leucocytopsermia. This would suggest that either ureaplasma infection does not induce a severe inflammatory response or the organism is a commensal. Subclinical genital tract infections in asymptomatic patients are unable to mount sufficient inflammatory response and thus do not play a major role in male infertility (Section 3.2). In the general infection group, the CD4/CD8 ratio was 1.8 in the infection positive group, in comparison to 1.9 in the infection negative group.

There was no increase in the CD14 and CD16 cells in the oligospermic group when compared to the normospermic group. This would suggest that these leucocyte subpopulations are not a major factor in influencing sperm structure (Section 3.3). In the oligospermic group, the CD4/CD8 ratio was 2 in the mild and in the control group, in comparison to 2.5 in the severe oligospermic group.

This is the first study to demonstrate raised pan T cells (CD3) and B cells (CD20) in the asthenospermic group. The activation of a resting helper T cell causes it to release cytokines, in particular IFN which is known to affect sperm motility. Similarly, activation of resting T helper cells could stimulate the activity of B cells (CD20), the latter producing antibodies and

thus further decreasing sperm motility (Section 3.4). In the asthenospermic group, the CD4/CD8 ratio was 2.2 in comparison to 2 in the control group.

The T cells (CD3 and CD8), B cells (CD20) and large granular lymphocytes (CD56) were significantly raised in the Oligoasthenospermic group, which shows that there exists a balance between T&B lymphocytes on sperm parameters. A disturbance or trigger factor could explain the recruitment of the lymphocytes to enhance sperm damage (Section 3.5). In the oligoasthenospermic group, the CD4/CD8 ratio was in 2.18 in the mild and 1.78 in the severe in comparison to 2 in the control group.

CD3 cells were significantly elevated in the germ cell damage azoospermic group. In Obstructive azoospermia, the T cells (CD3), B cells (CD20), large granular lymphocytes (CD56) and activated T and B cells (CD 69) were significantly raised. This might suggest that either the site of seminal leucocyte production is not necessarily confined to the vas or the epididymis as once thought or there is an increase in the T cell quantity being secreted as a result of an immune response to obstruction. Leucocytes may, in this situation, be secreted by the prostate and/or seminal vesicles (Section 3.6). In the azoospermic group, the ratio was 2.05 in obstructive and 4.71 in the germ cell failure, in comparison to 2 in the control group and a high CD4/CD8 ratio might be helpful in differentiating azoospermia secondary to germ cell failure.

The presence of leucocytes does not adversely affect the fertilisation rates and the outcome of an IVF cycle. However, the macrophages and the monocytes (CD14) were significantly elevated in the good fertilisers group in comparison to the poor fertilisers. This could be due to the fact that in these individuals there is increased phagocytic activity, which would

therefore remove the sperms with abnormal morphology and thus increase the fertilising potential (Section 3.7).

The CD4 / CD8 ratio was much lower in the ASA + ve group. The sperm numbers were also found to be low in this group. This might suggest that the CD8 lymphocytes actually have a more cytotoxic role: this would also correlate with the values from the germ cell failure group, as the ratio here was 4.71, which is at least double the value of all the groups. This would suggest low cytotoxic cell numbers, as there are no sperm to phagocytose.

Cytokines

IL-10, the anti-inflammatory cytokine, was significantly raised in the ASA +ve group. Raised IFN-γ was noted in the subfertile group, irrespective of the ASA status. This would imply that previous theories of how ASA could affect sperm motility in a mechanical way by antibody binding to the sperm tail may not be wholly true and that the cytokine IFN-γ could have had a role to play (Section 4.1).

There were no significantly elevated cytokine levels in the culture +ve group. This would tend to suggest either that the presence of asymptomatic infection might not produce the desired inflammatory response including the production of cytokines, or that the distal urogenital tract is not sterile (Section 4.2). Due to the lack of positive patients in my study, I am unable to either support or refute the role played by *Chlamydia trachomatis, Mycoplasma Species and Trichomonas Vaginalis* infections in male subfertility (Section 4.2).

Significantly elevated levels of IL-6 correlates with decreased sperm count and motility by its possible effect on sertoli cell transferrin production and meiotic DNA synthesis in the spermatocytes (Section 4.3). Although significantly raised levels of IL-8 correlated with

decreased sperm motility, its role in spermatogenesis and steroidogenesis has not yet been fully elucidated (Section 4.3). IL-10 was also significantly raised in the subfertile groups; this would suggest that it plays an anti-inflammatory role and tries to suppress the effects of other proinflammatory cytokines. This was confirmed by noticing significant correlations between IL-10 and other proinflammatory cytokines such as IL-6, IL-8 and TNF- α in the obstructive azoospermic group, and raised levels of IL-10 were found, along with raised levels of IL-6 & IL-8, in the asthenospermic group (Section 4.3).

IL-11 and IL-12 do not influence spermatogenesis (Section 4.3). TNF-α and IFN-γ levels were significantly increased ($p<0.05$) in the azoospermic obstructive group only. This would suggest that the testis is unlikely to be the site of production of these cytokines (Section 4.3). Not many cytokines had significant negative correlations with sperm parameters, which could suggest that the cytokines may not be originating in the testis and thus may not be influencing spermatogenesis. There was a significant correlation between the levels of IL-8 and IL-6. IL-6 is a multifunctional cytokine and it is possible that there exists a balance between IL-6 and IL-8 levels in the seminal plasma and that any alteration in this balance may result in male subfertility (Section 4.3).

Six out of the seven cytokines studied did not affect sperm-oocyte interaction and fertilisation rates in IVF. However, it appears that IL-11 could have a role in the fertilising capacity of the sperm and in preimplantation embryo development (Section 4.4). The washing of sperm as a part of the process of sperm preparation during IVF removes the seminal plasma from the sperm, and thus any potential adverse effect of cytokines on sperm capacitation and sperm-oocyte interactions.

Leucocytes & Cytokines (Chapter 5)

IL-6 correlated positively with activated T & B cells in the ASA positive group: it is likely that CD3 induces CD20 to release both IL- 6 and antibodies. IL-8 positively correlated with macrophages and monocytes (CD 14) in the culture positive group, as shown. CD14 is likely to mediate the induction of IL-8, which leads to inflammation. Thus, IL-8, either alone or in the presence of leucocytes, could drive the lipid peroxidation of the spermatozoa plasma membrane to levels that can affect the sperm fertility capacity.

I was unable to detect any correlation between the TNF- α and the leucocyte subpopulations studied. The results of my study indicate that there is a marked relationship between some pro-inflammatory cytokines and leucocytes, which affects semen quality. The significant association with seminal leucocytes and other potential inflammation markers suggests that IL-8 might be used as a sensitive marker for silent male genital tract infection.

6.1.3 Discussion

The significantly higher frequency of sperm morphological defects in infertile men may suggest that leucocytospermia as an independent variable has a detrimental effect on sperm morphology. A causal relationship between leucocytospermia and sperm morphology could be confirmed, if there is an improvement in sperm morphology after successful treatment of leucocytospermia. However, it may be difficult to conduct such a study given that treatment of leucocytospermia still remains controversial, as the presence of leucocytospermia is not always associated with bacterial infection or vice versa. This study would validate the above statement. Moreover, functional and anatomical damage acquired as a result of infection is often permanent and not reversible by (antibiotic) treatment. Hence, current evidence would suggest that the use of antibiotic therapy should only occur in men with symptomatic

leucocytospermia with microbiological evidence of bacterial infection. Maybe, instead of antibiotic treatment, the use of anti-inflammatory medications (particularly those with mast cell stabilising effects) may have a role to play in men with leucocytospermia and infertility, as some uncontrolled studies have shown a significant decrease in the leucocyte count and an improvement of sperm morphology and motility.

This study attempts to clarify not only the possible association between leucoyte concentration and sperm morphology / motility, but also the possible fertility-reducing effect of leucocytes. From this it is possible to speculate that sperm structural damage could be due to both defective Sertoli cell function and disorganized spermiation, or due to alteration in the maturation process that the sperm undergo while transient in the epididymis. Thus, leucocytospermia-induced sperm damage may commence during spermiogenesis and continue through spermiation and epididymal migration. This sperm damage could be mediated either through cytokines which have an influence on the sertoli cell function or by the peroxidative damage of ROS released by the activated granulocytes. To clarify the above issues, new methods for separation of leucocytes from other cells are needed. Future studies are needed, with large patient series and adequate follow-ups, concentrating on the role of reactive oxygen species in male subfertility and the relation between leucocytospermia at different leucocyte concentrations and fertility.

This study not only confirms the effect of the individual cytokines on sperm numbers and motility, but also shows that there is an intricate network of cytokines that exists in seminal plasma, which may influence sperm function directly or indirectly. It is possible that they affect the reproductive system differently through their soluble receptors[309] and could be upregulated in the presence of infections. This study's findings suggest that various cytokines

may work in an interactive manner that involves cross-talk through a network, to regulate fertility positively or negatively. The modulation of this cytokine network could explain the wide variability of the success rates seen in IVF when the male factor is ruled out by normal semen analysis.

IL-11 belongs to a family of cytokines whose receptors use gp130 as the signalling molecule (Chapter 1, Section 1.6.5). IL-11 has the ability to stimulate the multiplication of a variety of cell lines[310]. IL-11 is expressed in the round spermatids at stage VI-IX seminiferous tubules as shown[311] in Fig 6.1. IL-11 not only accelerates spermatogenesis but also has been shown to help in the recovery of spermatogenesis post cyotoxic therapy[311]. IL-11 reaches the uterus via the seminal plasma and mediates its actions via its receptor IL-11 R. Within the uterus, IL-11 has been shown to increase adhesion of primary human endometrial epithelial cells to fibronectin and collagen IV (Chapter 1, Section 1.6.5). It has been shown in murine models that defects in IL-11 signalling or lack of its receptor IL-11 R results in foetal loss due to defective decidualisation[312]. Furthermore, *in vitro* studies have shown that IL-11 may help with the implantation by reducing the quantity of endometrial TNF- α in early pregnancy[313]. Thus, IL-11 is not only an important regulator in testicular function by accelerating spermatogenesis and sperm recovery, but also helps with the endometrial decidualisation via its receptor, and helps in the implantation of the foetus as shown in Fig 6.1.

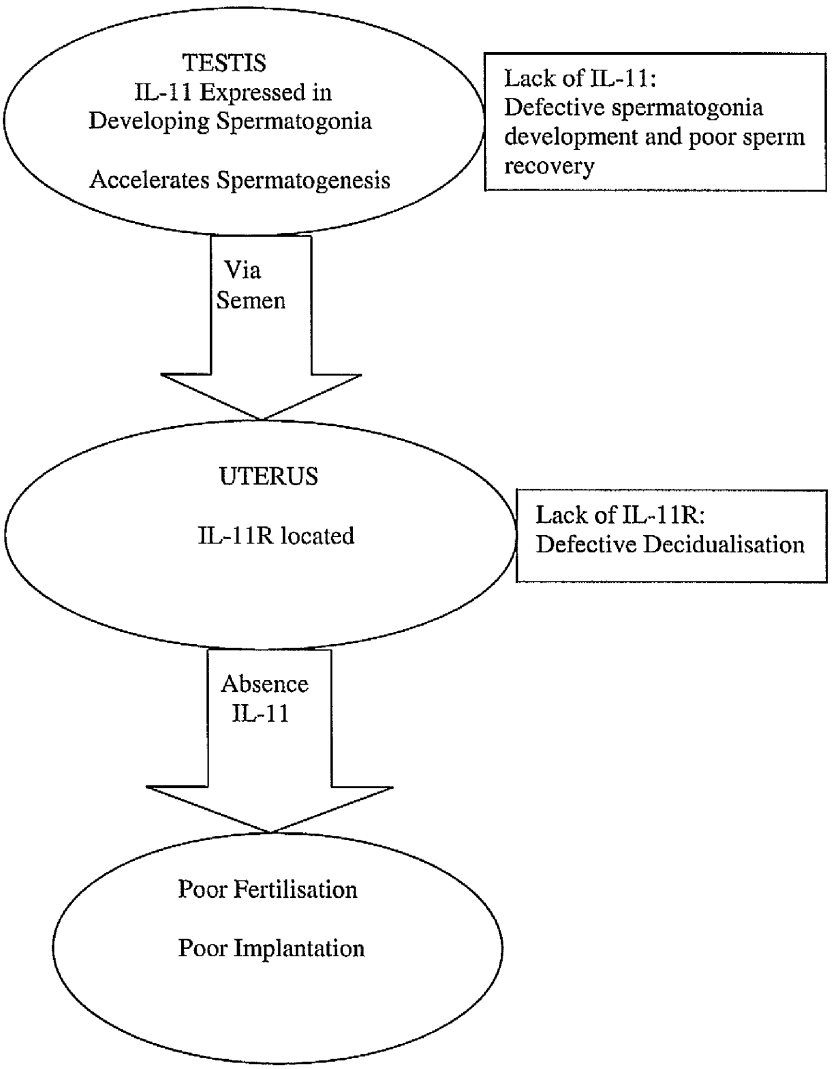


Fig- 6.1: Schematic diagram depicting the expression of IL-11 in the spermatogonia within the testis. The figure depicts the importance of the signal transduction by the seminal IL-11 via the endometrial receptor to help with the decidualisation and implanataion of the foetus.

However, further studies are required to assess the role of this cytokine network and immunomodulation in sperm function and transport, and particularly to answer such questions as: "If ICSI is used to get good fertilisation rates, could subsequent implantation be affected if cytokine concentrations are not right?" The measurement of each cytokine separately in the seminal plasma of subfertile men, despite significant differences, does not have a diagnostic value in male infertility. However, an assay of selective Interleukins in the seminal plasma of men may prove of clinical value for the diagnosis of male accessory gland infection.

It would be fair to conclude that neither leucocytospermia on its own nor individual cytokines have any significant role to play in male subfertiltiy. But the combination of leucocytospermia in the presence of a cytokine network may adversely affect sperm parameters and lead to male subfertility.

6.1.4 Limitations of the study-

There are a few limitations to our study. The numbers of patients in each group are small. Hence, statistical significance may not correlate with clinical significance. In our study we did not exclude patients who smoked and consumed alcohol, as none of them were heavy smokers (>20 cigarettes / day) {may increase the seminal leucocyte count and thus increase the seminal oxidative stress or decrease in semen concentration[314]} nor chronic alcoholics. {heavy alcohol use has been associated with an increase in the seminal leucocytes and chronic alcohol consumption (6 units / day for more than 5 days a week) has been shown to affect male reproductive hormones and semen quality}.[315]

Although we have endeavored to classify our patient sub-groups in reference to the 1999 WHO manual, some conventional authors may not agree with the quoted reference ranges of semen parameters. A revised definition was published by WHO in 2010. However, it is unlikely that the 2010 definition would have changed much of our results or interpretation. Progressive mean analysis could have been used to ensure whether 10HPF is good or not and to test statistical stability[316].

Some authors may not agree with our selection of markers used to detect leucocyte subpopulations. Although no significant different levels were detected in our study, some authors would have wanted us to test for the expression of CD 14 by the human germ cells. Correlations between CD 14 and L243 may be considered trivial, as monocytes/macrophages express MHC II (clone L243). CD 16 is not restricted to granulocytes and some pro-inflammatory monocytes express CD14 and CD16.

The total leucocyte concentration was represented by CD45 count. However, I did not look at the total leucocyte count obtained during semen analysis. IL-1 was not studied in depth as it has been studied extensively in the past, and, furthermore, has three members in the family, two of which are proinflammatory and the third has an anti-inflammtory effect. It would have been useful to look at the number of biochemical/clinical pregnancies resulting from the embryos created by the good and poor fertilisers. This would have then led to further discussion about the correlation / relationship between pregnancy outcome and the seminal plasma cytokine content.

6.1.5 Future research projects-

- Performing testicular biopsies on patients with impaired sperm parameters for the presence of inflammatory infiltrates, which would further strengthen my study findings.

- A study into the role of cytokines in the oocyte / embryonic culture medium, as this would increase the relevance of my study.

- The role of IL-11 in implantation would be useful to look at, as it was elevated in the good fertiliser group. Furthermore, it has been shown in murine model that decidual and feotoplacental development is dependent on IL-11.

- The role of Ketotifen in Leucocytospermia- RCT, as some cohort studies have shown that anti-inflammatory medication such as ketotifen reduces leucoyte count and improves semen quality.

- The role of thymus extract in male subfertility and the role of leucocytes and cytokines in recurrent miscarriages may be other useful future research topics.

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APPENDICES
Appendix 1 (Discussed in Section 2.1)

THE UNIVERSITY
of LIVERPOOL

Patient Information Sheet

Leucocytes and cytokines content of human semen including males attending an In Vitro Fertilisation (IVF) programme

What is the purpose of this study?

White cells (called leucocytes) and chemical substances (called cytokines) are normally present in all semen samples but at a very low concentration. Previous reports have revealed that the presence of these in high concentrations in semen could affect the sperm and their ability to fertilise eggs.

The aim of our study is to evaluate whether or not increased numbers of leucocytes and increasing quantitiy of various cytokines in semen influence sperm parameters and their fertilising potential. Once we know how leucocytes and cytokines affect fertility, we may be able to provide a treatment to help patients who have an excess of leucocytes in their semen. We hope that the results of our study will give us a better understanding of this subject and enable us to help people who are infertile due to this condition.

What will happen to me if I take part?

If you give permission after routine semen analysis, we will use any remaining sample for research purposes and those who are in an IVF programme it does not affect your success rate.

Who is organising the study?

The study is organised by Dr. Srividya Seshadri, Research Fellow in Obstetrics and Gynaecology, Liverpool Women's Hospital. It is a combined work between the Departments of Obstetrics and Gynaecology and Immunology of the Liverpool University.

Confidentiality

All information collected about you during the course of study will be strictly confidential. Any information about you that leaves the hospital will be anaonymised so that you cannot be recognised from it. Your identity will therefore not be revealed even if the results of the study are published. We would usually expect to inform your GP that you are participating in the study. Please let us know if you prefer us not to do this.

Voluntary Participation:

Participation in this study is voluntary. If you would like to join the study please complete the attached Consent Form. If, at any time you wish to withdraw from the study there is no need to justify your decision and your future medical treatment will not be affected.

Contact:

If you have any questions or wish further information at any point in study, contact:

Dr S. Seshadri
Liverpool Women's Hospital
Crown Street
Liverpool
L18 7SS
Tel: 0151 708 9988 Ext 4215

Thank you for reading this information and agreeing to take part in this study

Appendix 2 (Discussed in Section 2.1)

CONSENT FORM

Title of the project: Leucocytes and cytokines content of human semen including males attending an In Vitro Fertilisation (IVF) programme

Name of the Researcher: Dr Srividya Seshadri

[Name and number of independent person]

Please initial box

1. I confirm that I have read and understood the information sheet for the above study, including the use of surplus sample that remains after semen analysis.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without medical care or legal rights being affected.

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from (company name) or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

4. I agree to take part in the above study

Name of patient	Date	Signature
Researcher	Date	Signature

1 copy for patient, 1 copy for researcher, 1 copy to be kept with hospital notes

Appendix 3

Semen Analysis Report

Reproductive Medicine Unit, Liverpool Women's Hospital

SEMEN ANALYSIS REPORT

Female name ..... Hospital no .....

Male name ..... Hospital no .....

Date of analysis .....D.O.B.....Lab code no .....

GOPD IVF-NHS IVF-PRI ADD-NHS ADD-PRI OTHER Referring clinician .....

Time of sample production ..... Ejaculation - analysis interval ....mins

Appearance ..... Liquefaction: *Complete* *Incomplete*

pH ..... Round cells ..... x 10^6/ml Debris 0 (+) ++ +++

Volume ..... mls

Concentration ..... x 10^6/ml

Morphology ..... % normal forms

Antisperm antibodies (ASAb)

IgG ..... % binding

IgA ..... % binding

Agglutination 0 (+) ++ +++

Motility at ..... °C

Grade A ..... % *(excellent progression)*

Grade B ..... % *(sluggish progression)*

Grade C ..... % *(non-progresstive)*

Grade D ..... % *(non-motile)*

Viability ..... % viable

Comments

Analysis performed by

SPERM MIGRATION TEST RESULT

Final concentration

..... 0^6/ml

Motility at ..... °C

Grade A ..... % *(excellent progression)*

Grade B ..... % *(sluggish progression)*

Grade C ..... % *(non-progresstive)*

Grade D ..... % *(non-motile)*

REFERENCE VALUES

(WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction, 4th edition, 1999)

Volume: ≥ 2.0mls
pH: ≥ 7.2
Concentration: ≥ 20 x10^6/ml
Motility: ≥ 50% grades A + B or ≥ 25% grade A within 60 mins of ejaculation

Morphology: ≥ 15% *
Viability: ≥ 75%
ASAb: < 50%
Round cells: ≤ 5 x10^6/ml

**Data suggest that as sperm morphology falls below 15%, fertilisation rate in vitro decreases.*

Appendix 4

Preparation of slides

Materials required:

-Bovine serum albumin (BSA; Sigma, Poole, U.K.)
-Trypan blue
-Teflon coated multiwell slides (C.A.Hendley Ltd. ESSEX)
-Acetone
-Phosphate buffered saline (PBS);

$CaCl .H_2 O$	0.132 g
KCl	0.2g
KH PO	0.2g
MgCl 6H O	0.1g
NaCl	8.0g
Na HPO	1.15g

Make up to 1 litre with distilled water.

Preparation of solutions

Tris buffer saline (TBS)

1) TRIS (molecular weight:121.1x molarity 50 mM= 6g/l) weigh 30 g/5L
2) NaCL (molecular weight:58.44 x molarity 150 mM= 8.7g/l) weigh 43.59 g/5L
3) Dissolve in 1.5 L distilled water, and stir
4) Adjust pH 7.6 using Hydrochloric acid (11.6 M)

Add distilled water upto 5 L and mix.

0.1M Phosphate buffer saline (PBS) (Sigma D-5773)

Magnesium Chloride (anhydrous)	0.046g/l
Potassium Chloride	0.2 g/l
Potassium Phosphate Monobasic (anhydrous)	0.2 g/l
Sodium Chloride	8.0 g/l
Sodium Phosphate Dibasic	1.15 g/l

Adjust Ph 7.6 using Hydrochloric acid (11.6 M)

Alkaline phosphatase substrate

1) 0.1 M Tris buffer pH 8.2	50 mls
2) Naphthol AX-MX phosphate (Sigma N-4875)	10mg
3) Dimethyl formamide (sigma D-4254)	1.0 ml
4) Levamisole (sigma L-97560) 1M solution	50 µl

Mix in glass bottle, store at 4°C.

0.1 M Tris buffer pH 8.2

Weigh 6.055gm and add 500ml distilled water adjust to pH 8.2 using hydrochloric acid (11.6M)

10% BSA (bovine serum albumin) (Sigma A- 2153)
Dissolve 1gm BSA in 10 ml TBS buffer solution, aliquot in microfuge tubes, and store in freezer at -20°C.

Preparation of antibody

Initial solution of 10mls TBS added to 0.5ml (0.5%) BSA
Appropriate amount of antibody added to 1ml of solution depending on dilution used

APAAP

Dilute APAAP complex 1:50 in TBS

Fast Red

10mg of fast red diluted in 10ml of alkaline phosphatase passed through filter paper.

Appendix 5-
Publications

1) ***Citation- Seshadri et al. The Role of Cytokine Expression in Different Subgroups of Subfertile Men. AJRI 2009; 62 (5): 275-282.**

2) **"Cytokine expression in the seminal plasma and its effects on fertilisation rates in an IVF cycle". *This study has been accepted for publication in the Andrologia Journal.**

3) **"Detection of subpopulations of leucocytes in different subgroups of semen sample qualities"- This study has been accepted for publication in Andrologia Journal**

Presentations

Poster :

1. "The role of leucocyte subpopulations in male subfertility" June 2010 ESHRE, Rome. Hum. Reprod. (2010) 25(Suppl): i118-152

2. "Does Ureaplasma play a role in leucocytospermia or male infertility?" 8th RCOG International Scientific Meeting, December 2009, Abu Dhabi

3. "The role of cytokine expression in different subsets of subfertile men". ASRM, New Orleans. Fertil Steril, (2006) 86 (Supp2) : S492-S493

4. "Cytokines as positive predictors of fertilisation rates in an IVF cycle" - December 2003, British Fertility society, Liverpool. Runner up for the best poster presentation.

5. "The effect of cytokine expression in the seminal plasma on the fertilisation rates in an IVF cycle". October 2003, ASRM Conference, San Antonio, Texas. Fertil Steril (2003) 80 (Supp 3): S210.

Oral:

1. "Leucocytospermia and IVF outcome" – (BFS/ACE/SRF/ICE/IFS) Joint Fertility, January 2011, Dublin.

2. "Cell Mediated Immunity in Male subfertility"- Annual BFS Meeting January 2010, Bristol.

3. "The role of cytokine expression in azoospermia" –4th Joint BAS/BFS/SRF Meeting April 2005, Warwick.

The Role of Cytokine Expression in Different Subgroups of Subfertile Men

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Problem
The aim of this study was to evaluate the levels of seminal plasma cytokines interleukin 6 (IL-6), interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 11 (IL-11), interleukin 12 (IL-12), tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) in male subfertility.

Method of study
A total of 73 male partners of an infertile couple attending a regional andrology unit were recruited into this prospective study and subdivided into the various groups based on semen analysis. Concentrations of cytokines such as IL-6, IL-8, IL-10, IL-11, IL-12, TNF-α and IFN-γ in the seminal plasma were determined using enzyme linked immunosorbent assay (ELISA).

Results
Significant higher concentrations ($P < 0.05$) of IL-6 in the mild and severe oligospermic group, IL-8 and IL-10 in the asthenospermic group and IL-6, IL-10, TNF-α and IFN-γ in the obstructed azoospermic group were determined. IL-10 concentrations correlated significantly with other cytokines in the obstructed azoospermic group and the asthenospermic group.

Conclusion
Our study confirms that cytokines rarely act in isolation, but rather in a network of other cytokines and may affect sperm function directly or indirectly. The presence of increased levels of cytokines in the obstructed azoospermic group suggests that the cytokines may not originate from the testis.

Introduction

Male factor infertility is considered to be responsible as the primary cause or a contributory factor in up to 45% of infertile couples.[1] Cell-mediated immunity through cytokines may play a key role in male factor infertility.[2] Most cytokines act locally to initiate and modulate immune responses, principally by binding to specific, high affinity receptors on responsive cells.[3] Some exert their effects distally in an endocrine manner.

Spermatogenesis is an autonomous process largely under the control of paracrine factors. Cytokines that potentially affect cell populations involved in

spermatogenesis are produced within the seminiferous epithelium and influence the post-meiotic stages of spermatogenesis. The role of these paracrine regulatory proteins remains to be elucidated. Cytokines have also been implicated in the regulation of growth and differentiation of cells in the endocrine and tubular compartment of the testis. Paracrine regulation of cellular interactions in the testis is an integrated system between hormones and the local environment[4]. Cytokines are important factors in the integration of the neuroendocrine-immune network that controls testicular function. The predominant consensus is that tumour necrosis factor alpha (TNF-α), interleukin 1 (IL-1), interferon gamma (IFN-γ) and interleukin 2 (IL-2) inhibit gonadotrophin or cyclic adenosine mono phosphate (cAMP) stimulated steroidogenesis in Leydig cells.[5] Inflammatory disease has been established as detrimental to male reproductive function and fertility. Infections of the male accessory glands appear to affect reproductive function as indicated by decreased density and motility of spermatozoa and alterations in seminal plasma constituents. Human spermatozoa incubated with IFN-γ and TNF-α have demonstrated significantly reduced motility. In addition, both cytokines compromised the fertilizing ability of the spermatozoa as determined by the zona free hamster egg penetration test.[2] Elevated levels of cytokines in the seminal plasma of men with fertility problems have been clearly documented.[6–8] Recently, sperm concentration, motility and the percentage of normal sperm forms were found to be decreased with increasing interleukin 6 (IL-6) levels in seminal plasma.[9] Previously, elevated levels of interferon alpha (IFN-α) and IFN-γ have been found in patients suffering from oligospermia and azoospermia.[10]

The objective of this study was to determine the relationship of a repertoire of cytokines in the seminal plasma of different subgroups of subfertile men attending a regional andrology clinic. To the best of our knowledge, there have been no studies testing these seven cytokines (IL-6, IL-8, IL-10, IL-11, IL-12, TNF-α and IFN-γ) in the different subsets of male subfertility.

Material and methods

Study Design

A prospective comparative study was designed in which semen was collected from men attending the Reproductive Medicine Unit of the Liverpool Women's Hospital for infertility investigation. The median age of men was 34.5 (range 22–49) years. The medium duration of infertility was 5 (range 2–13) years. Thirty-eight of the men consumed alcohol on an average of 10–12 units/week; 20 of the men also smoked on an average of 10–20 cigarettes/day. Men were divided into the different subgroups (normospermic, asthenospermic, mild oligospermic, severe oligospermic, mild oligoasthenospermic, severe oligoasthenospermic, obstructive azoospermic and non-obstructive azoospermia) according to the results of the seminal analysis on two consecutive semen samples. The seminology laboratory at the hospital carried out standard sperm function tests on each sample, including details on sperm number, motility and the presence of bound anti-sperm antibodies etc., as detailed by WHO. These men had no other illnesses nor did they take any medication that could have affected their cytokine metabolism.

Ethical approval was granted by the Liverpool Research Ethics Committee. Written, informed consent was obtained from 73 men attending the Reproductive Medicine Unit, Liverpool Women's Hospital. Clinical details of both partners were obtained from the case notes.

Semen Collection

Semen was obtained by masturbation after 3–5 days of sexual abstinence. The samples were collected in a sterile, wide mouthed, non-toxic container and processed in the laboratory within 1 hr of ejaculation. Samples did not get exposed to extremes of temperature. They did not come in contact with either lubricants or latex products. All the samples underwent semen analysis in the laboratory in accordance with the 1999 WHO laboratory manual for the examination of human semen and the study was performed using these reference values.[11] Men were divided into the different sub groups according to the results of two consecutive semen analysis 6 weeks apart. Given the wide variability between ejaculates, two samples were taken to confidently define an individual's phenotype. Asthenospermia was defined as a sperm motility of <40%. Oligospermia was defined as a sperm count <10–20 × 10^6. Oligoasthenospermia was defined as a sperm count of 10–20 × 10^6 and motility of <40%. Azoospermia was defined as complete absence of sperm in the semen either as a result of germ cell failure such as

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maturation arrest, hypospermatogenesis or sertoli cell only syndrome (non-obstructive group) or as a result of obstruction in the sperm delivery system. The obstructive azoospermic group had all undergone vasectomy. Although testicular biopsy is the definitive test to rule out the non-obstructive from the obstructive groups, the presence or absence of active spermatogenesis can be accurately predicted by measuring the testis volume and serum follicle stimulating hormone (FSH).[12] Hence the differentiation between germ cell failure azoospermia and obstructive azoospermia was made by the serum FSH levels, karyotyping and testicular volume. The testicular volume in our non-obstructive group was reduced (<7 mL). After semen analysis, the remaining sample was processed for the measurement of cytokines.

Preparation of Seminal Plasma

Samples were centrifuged at 200 × *g* for 10 min and the supernatant removed and immediately stored at −20°C until assayed to prevent the effect of proteases on the individual cytokines. The samples were assayed in the same run to reduce inter assay variability. Cytokine concentrations were measured using specific commercial ELISA kits (TNF-α by Genzyme Duoset Kit, Cambridge, MA, USA and IL-6, IL-8, IL-10, IL-11, IL-12 and IFN-γ from R&D Systems, Abingdon, UK). The range of measurement of the cytokines was IL-6 (3.125–300 pg/mL), IL-8 (31.2–2000 pg/mL), IL-10 (31.25–2000 pg/mL), IL-11 (15.6–1000 pg/mL), IL-12 (7.8–500 pg/mL), IFN-γ, TNF-α (15.6–1000 pg/mL). The sensitivity of the assays for IL-6, IL-8, IL-10, IL-11, IL-12, IFN-γ and TNF-α was less than 0.6, 2.6, 6.3, 4.4, 2, 8 and 1.6 pg/mL respectively. The inter and the intra-assay variability was 12.2% and 0.53% for IL-6, was 1.4% and 2.94% for IL-8, was 15.3% and 1.32% for IL-10, was 49.0% and 1.46% for IL-11, was 24.2% and 0.64% for IL-12, was 7% and 1.63% for TNF-α and 14.4% and 1.69% for IFN-γ.

Statistical Analysis

Statistical analysis was performed using the statistical package for social sciences (SPSS, Chicago, IL, USA) software. The Shapiro–Wilkes test was used to test for normal distribution. The individual cytokine levels in the normospermic group were compared with the levels of the respective individual cytokine from the other subsets. As our data were not normally distributed, statistical analysis was performed using the Mann–Whitney non-parametric two-sample test. Correlations between cytokine concentrations were tested using the non-parametric Spearman's correlation test. The number of patients is relatively small, as this was aimed to be a pilot study and if significant levels of cytokines were found in the various subgroups then an adequately powered study will be required to determine both the statistical and the clinical significance of these findings.

Results

The normospermic group was compared with each of the other patient subsets. The median values and the ranges for all the cytokines are shown in the Table I. Significantly higher concentrations of IL-6 ($P < 0.05$) were present in the oligospermic, asthenospermic, oligoasthenospermic, azoospermic obstructive and germ cell damage groups when compared with the normospermic group. Significantly higher concentrations of IL-8 ($P < 0.05$) were present in the asthenospermic group when compared with the normospermic group. Significant amount of IL-10 ($P < 0.05$) was present in the asthenospermic, oligoasthenospermic and azoospermic obstructive groups when compared with the normospermic group. Furthermore, IL-6, IL-10, TNF-α and IFN-γ were significantly increased in the obstructive azoospermic group when compared with the normospermic group. A significant correlation was found between IL-10 and IL-11 in the oligospermic ($P < 0.05$) and obstructive azoospermic ($P < 0.01$) groups. A significant correlation ($P < 0.01$) was found between IL-10 and IL-11 and between IL-10 and IFN-γ in the oligoasthenospermic group (Fig. 1). This is shown to allude to the fact that cytokines can affect both the sperm numbers and motility. A significant correlation ($P < 0.05$) was found between IL-10 and IL-6 in the oligoasthenospermic group. A significant correlation ($P < 0.01$) was found between IL-10 and IFN-γ in the oligospermic and asthenospermic groups.

Discussion

During the last decade, there has been much evidence to suggest the involvement of paracrine control mechanisms such as the cytokines, in the control of reproductive function particularly with regard to spermatogenesis.[13] Cytokines may have a

Table 1 The Median and the Ranges of Various Cytokines (pg/mL) in Different Patient Subgroups are Shown (*$P < 0.05$)

Cytokine levels (pg/mL)	IL-6	IL-8	IL-10	IL-11	IL-12	TNF-α	IFN-γ
Normospermic (n = 14)	18 (4–73)	1300 (220–2600)	76.5 (0–357)	6472 (13–11,760)	21.5 (0–100)	0 (0–44)	45 (97–2135)
Asthenospermic (n = 8)	69.5 (4–568)*	2850 (450–49,500)*	158 (35–832)*	4602.5 (750–27,417)	11 (0–26)	0 (0–0)	563 (192–1665)
Oligospermic (n = 13)	38 (8–164)*	1450 (250–2500)	78 (39–175)	5815 (2550–14,083)	6 (0–72)	0 (0–41)	608 (2–1551)
Oligoasthenospermic (n = 19)	31 (7–355)*	1750 (160–32,750)	122 (0–245)*	4125 (333–16,980)	0 (0–110)	0 (0–51)	629 (59–1566)
Azoospermic obstructive (n = 10)	42 (14–101)*	2050 (1250–5000)	186 (80–490)*	9529 (3750–21,804)	25 (6–140)	23 (0–73)*	1049.5 (202–1704)*
Azoospermic non-obstructive (n = 9)	37 (11–1006)*	2100 (700–3250)	93 (2–253)	4991.5 (0–33,441)	18 (0–80)	0 (0–14)	901 (0–1575)
Normospermic (n = 14)	18 (4–73)	1300 (220–2600)	76.5 (0–357)	6472 (13–11,760)	21.5 (0–100)	0 (0–44)	454 (97–2135)
Asthenospermic (n = 8)	69.5 (4–568)*	2850 (450–49,500)*	158 (35–832)*	4602.5 (750–27,417)	11 (0–26)	0 (0–0)	563 (192–1665)
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Azoospermic (n = 20)	30 (11–1006)*	2000 (1000–41,00)*	91.5 (83–490)**	5200 (0–52,083)	10 (0–140)	2.5 (0–73)	1017.5 (202–1704)

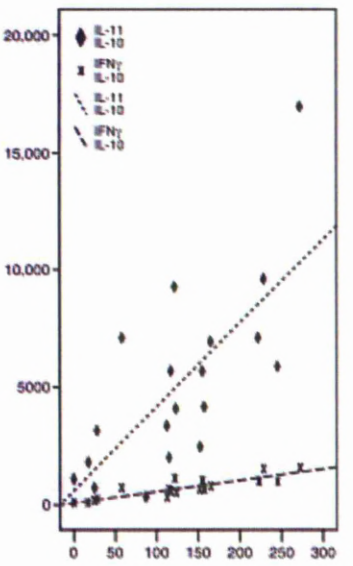

Fig. 1 Correlation between IL-10 with IL-11 and IFN-γ (pg/mL) in the oligoasthenospermic group.

multitude of actions including both as growth and differentiation factors within the seminiferous tubule. IL-6 is produced by the Sertoli cells and has a number of effects including stimulation of transferrin production by sertoli cells[14] and inhibition of meiotic DNA synthesis in pre-leptotene spermatocytes.[15]

In this study, statistically significant raised levels of IL-6 were found in the oligospermic, asthenospermic and the oligoasthenospermic groups. These results correlate with two previous studies where increased levels of IL-6 were found in the infertile groups.[9,16] Similarly elevated levels of IL-6 have correlated significantly with sperm number in the ejaculate, penetration rates in the sperm penetration assay, and some sperm motion parameters.[6] Increased levels of IL-6 were found in men with accessory gland infection when compared with normal men, but in contrast to our study, IL-6 levels did not show any significant difference between normal men and those with abnormal spermiograms as, the testis may not contribute to the high IL-6 levels in the seminal plasma[17] but may be coming from

the seminal vesicles and/or prostate. Similarly significant correlations of IL-6 and fructose levels indicated that the seminal vesicles also take part in the production of seminal IL-6.[18] In our study, there were significantly high levels of IL-6 in the obstructive azoospermic patients.

In this study, IL-8 ($P < 0.05$) was significantly increased in the asthenospermic group when compared with the normospermic group. This may be due to the fact that asthenospermics may have epididymal disease secondary to a male accessory gland infection. The origin of IL-8 and its role in the spermatogenesis and steroidogenesis has not been elucidated but investigations have revealed that there are significant differences in levels between normal and infertile men with leucospermia/male accessory gland infection.[19]

IL-10 was increased ($P < 0.05$) in the asthenospermic, oligoasthenospermic and azoospermic obstructive groups when compared with the normospermic group which may suggest that the testis is probably not the site of its production. A recent study also concurs with our study that IL-10 levels were significantly elevated in seminal plasma of men with decreased sperm motility.[20] In contrast, high levels of IL-10 have been observed in the seminal plasma of fertile men when compared with infertile men with or without leucocytospermia.[21] However, in their study the classification of infertile groups was solely based on leucocytospermia.

In our study, we found significant correlations between IL-10 and other proinflammatory cytokines in the oligospermic, asthenospermic, oligoasthenospermic and azoospermic groups. Raised levels of IL-10 were found along with raised levels of IL-6, IL-8 and TNF-α in the azoospermic obstructive group and raised levels of IL-10 was found along with raised levels of IL-6 & IL-8 in the asthenospermic group. IL-10 is an anti-inflammatory cytokine with immunoregulatory functions and inhibits the production of several proinflammatory cytokines including IL-8 by altering the accessory cell function of macrophages.[22] Hence it is possible that the raised levels of IL-10 observed in this study was in direct response to suppress the proinflammatory cytokines such as IL6, IL-8 & IFN-γ. In contrast, IL-10 levels have been shown to be significantly decreased in the seminal plasma of oligo-teratozoospermia and asthenoteratozoospermic patients with genital infection when compared with fertile, azoospermic or oligo-teratoasthenozoospermic patients without infection.[23]

Previously, increased levels of IL-11 were found in men with male accessory gland infection when compared with normal men, but IL-11 levels did not show any significant difference between normal men and those with abnormal spermiograms.[24] Our study, did not find any statistically significant difference in the levels of IL-11 amongst the various sub groups of subfertile men in comparison with normospermic group. This would suggest that this cytokine may not be influencing spermatogenesis.

This study demonstrated no significant difference in the IL-12 levels between the normospermic and subfertile sub groups. This is in agreement with other studies.[23,25]

Elevated levels of TNF-α and IFN-γ have been identified in the oligospermic and azoospermic groups.[10] TNF-α levels in seminal plasma have been shown to be negatively correlated with the number of progressively motile sperm in the ejaculate, but there was no correlation with total sperm counts, viability, morphological alterations etc.[26] TNF-α levels were significantly elevated in seminal plasma of men with decreased sperm motility.[20] Significantly increased levels of TNF-α were found in the oligoasthenozoospermic and the azoospermic groups when compared with the normospermic group.[27] However, the azoospermic group was not subdivided into obstructive and non-obstructive. In direct contrast, there was no relationship between TNF-α levels in seminal plasma and semen quality or parameters of sperm functional capacity.[28] However, in this study, the azoospermic men were excluded. IFN-γ is thought to stimulate the production of TNF-α and it is reported that there is a significant overproduction of IFN-γ levels in the seminal plasma of infertile men and a significant negative correlation with sperm count and motility.[29] IFN-γ levels were significantly elevated in seminal plasma of men with decreased sperm motility.[20]

In our study, the TNF-α & IFN-γ levels were significantly increased ($P < 0.05$) in the azoospermic obstructive group only. This would suggest that the testis is unlikely to be the site of production of these cytokines. Although some of the median values in the TNF-α measurement are 0 as it was the most frequently occurring number, looking at the mean values, the TNF-α levels in the obstructive azoospermic group was 27.8 in comparison with other groups where the mean values ranged from 0–5.1. Our study would support the finding of another study that TNF-α does not affect sperm numbers or

motility.[28] Prominent cytokines associated with T cell function, such as IL-10, IL-12 and IFN-γ, were detected at low concentration (< 100 pg/mL) in only a few samples of normal men. This is similar to our normospermic group values. This suggests that cellular immune activity is low in the genital tract of normal men.[30] This cell mediated immunity could be up regulated by genital tract infection and lead to an increase in the levels of these cytokines.[31]

As increased levels of cytokines were found in the obstructive azoospermic group, the measured levels must be coming from either the seminal vesicles or the prostate. Although unlikely, there may even be a small contribution from the epididymis. The only definitive way to prove that the cytokines are not originating from the testis is to measure the cytokine levels from testicular sperm extraction samples.

The pathophysiological significance of the cytokines in sperm function is still unclear and controversial. Cytokines rarely act in isolation, but rather in a network of other cytokines (e.g.) elevated levels of IL-6, IL-8 and IL-11 were found in the seminal plasma of infertile men with infection and oligo- terato-asthenozoospermia.[32] Hence we wanted to see if there was a significant correlation between the different cytokines in subfertile men. This study shows that there is an intricate cytokine network that exists in seminal plasma which may influence sperm function directly or indirectly. However, as the range of cytokine levels in our study is too large, it is difficult to define a cut-off value and thus suggest a clinical use for routine measurement of cytokine levels in subfertile men. Our study suggests the possible effect of the individual cytokines on sperm motility. It is possible that they affect the reproductive system differently through their soluble receptors.[33] Proinflammatory cytokines such as IL-6, IL-8, and TNFα modulate pro-oxidant and antioxidant activities in the semen and thus may directly influence semen parameters.[34] However, further studies are required to assess the role of this cytokine network and immunomodulation in sperm function and transport. The measurement of each cytokine separately in the seminal plasma of subfertile men, despite significant differences, does not have a diagnostic value in male infertility. However, an assay of selective cytokines and leucocytes in the semen of men may prove to be of clinical value in the diagnosis of male accessory gland infection and facilitate treatment.

There are a few limitations to our study. The number of patients in each group is small. Hence statistical significance may not correlate with clinical significance. In our study, we have not excluded patients who smoke and consume alcohol as none of them were heavy smokers (>20 cigarettes/day) or chronic alcoholics. Controversy exists about the role of smoking and male infertility. Some studies have shown that smoking has a detrimental effect on sperm quality. However, other studies have not found an association between smoking and sperm quality, sperm functions, or sperm nuclear DNA damage. Standard semen parameters were not different between infertile men who smoked and those who did not smoke.[35] In another study, heavy smoking was associated with a decrease in semen concentration.[36] It is likely that some authors may not agree with us, due to the fact that smoking may increase the seminal leucocyte count and thus increase the seminal oxidative stress. Alcohol is known to alter cytokine levels in a variety of tissues including plasma, lung, liver, and brain. Studies on human monocyte responses revealed that chronic alcohol use alters inflammatory cytokine production.[37] Chronic alcohol consumption (6 units a day for more than 5 days a week) has shown to affect male reproductive hormones and semen quality.[38]

Although we have endeavoured to classify our patient sub groups in reference to the WHO manual, some conventional authors may not agree with the quoted reference ranges of semen parameters. The measurement of seminal plasma leucocytes during male genital tract inflammation without an associated contribution of cytokines may have little prognostic value in the evaluation of male infertility. Hence seminal leucocyte concentrations were also measured in these sub groups. The median and the ranges of the total CD 45 amongst the various study groups are presented. The total CD45 counts between the normospermic group [5.9 (3.9–16.7)] and the other sub groups of subfertile men [oligospermia (8.4 (4–26.1) $P < 0.69$), asthenospermia (12.3 (9.6–18.4) $P < 0.39$), oligoasthenospermia (7.75 (4.1–12.4) $P < 0.945$) and azoospermia (5.5 (2.6–11.1) $P < 0.57$)] in our study were not found to be statistically different. It is of clinical relevance in this setting and our study concurs with the findings of a recent study where none of the cytokines involved in our study was associated with increased cellularity in the semen.[20] IL-1 was not studied in our study as it has been studied extensively in the past and furthermore, has three members in the

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family, two of which are proinflammatory and third has an anti-inflammatory effect.

To the best of our knowledge, no previous studies have concurrently analysed the simultaneous expression of these seven cytokines in the seminal plasma of men belonging to different groups of male subfertility. The effects of individual cytokine on sperm motility and numbers are also confirmed. The presence of increased levels of cytokines in the obstructed azoospermic group suggests that the cytokines may not originate from the testis. This study also highlights the presence of an intricate cytokine network in these subfertile men.

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"The detection of subpopulations of leucocytes in different subgroups of semen sample qualities"

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THE DETECTION OF SUBPOPULATIONS OF LEUCOCYTES IN DIFFERENT SUBGROUPS OF SEMEN SAMPLE QUALITIES

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ABSTRACT

The role of leucocytospermia in male subfertility is a much debated topic despite being a frequent finding. This study aimed to identify the role of leucocytes, leucocyte subpopulations and natural killer cells in male subfertility. 76 subfertile men attending a regional andrology unit were recruited into this prospective study and subdivided into groups based on their semen analysis. The different leucocyte subpopulations were identified using immunocytochemical staining. Significant levels of CD3 helper T lymphocytes ($p<0.001$) were present in the oligospermic, asthenospermic, oligoasthenospermic and obstructive azoospermic group compared to the normospermic group. Significant levels of B cells ($p<0.05$) were present in the asthenospermic, oligoasthenospermic and obstructive azoospermic group. The natural killer cells (CD56) were significantly raised in the oligoasthenospermic and obstructive azoospermic group ($p<0.05$). Our study suggests that leucocytospermia impairs sperm function through enhanced T helper cell modulation, increased B cell population which leads to increased levels of antisperm antibody and natural killer cells mediated sperm damage. The site of seminal leucocyte production is not necessarily confined to the vas or the epididymis.

Key words: leucocytes, sub fertility, sperm count, sperm motility

INTRODUCTION:

The relationship between leucocytes and the fertilising potential of an ejaculate remains highly controversial. Leucocytospermia is a frequent finding in infertile patients and is associated with poor semen quality. The incidence of leucocytes in the seminal fluid among infertile men, ranged from 15 to 28%, whereas in fertile men it is 10% (Zorn et al. 2000). Increased polymorphonuclear (PMN) concentrations are related to decreased percentages of normal forms of spermatozoa and a significant increase in the percentage of midpiece abnormalities (Thomas et al. 1997). Another study showed that, the patient group with lower mean leucocyte concentration ($0.5x10^6$ leucocytes per ml) had a significantly higher proportion of sperm with normal morphology compared with patients who had a higher mean leucocyte concentration. There was also a significant positive correlation between leucocyte concentration and midpiece defects (Thomas et al. 1997).

A potential mechanism through which leucocytospermia may induce alteration in sperm function is excessive reactive oxygen species (ROS) production by activated granulocytes. It has been debated that oxidative stress may induce alterations in the regulation of spermatogenesis, resulting in structural defects of the sperm (Gil-Guzman et al. 2001;Ollero et al. 2001). On the subcellular level, it has been shown that leucocytospermia and excessive ROS levels are associated with an increase in chromatin alterations and DNA damage in sperm, as defined by the sperm chromatin structure assay (Alvarez et al. 2002). A different study, has shown that a high incidence of sperm tail defects is associated with sperm chromosomal abnormalities (Lewis-Jones et al. 2003). It is thus conceivable that oxidative genetic damage may have led to a significant increase in the proportion of sperm with tail defects observed in leucocytospermic study population.

However some authors failed to prove this association between sperm morphology and leucocytospermia. One study even demonstrated leucocytospermia to have a positive association with sperm concentration; acrosome reaction and hypoosmotic swelling (HOS) test scores (Kaleli et al. 2000). Sperm concentration was found to be constantly higher at increasing leucocyte concentrations. Surprisingly, this study was also unable to document any significant effect on sperm motility, even when the leucocyte concentrations increased[7]. It was even suggested that leucocytes have a role in the removal of abnormal spermatozoa from the ejaculate, thereby increasing the percentage of sperm with normal morphology (Tomlinson et al. 1992). Other studies have not observed sperm damage during leucocytospermia (Kaleli et al. 2000).

These conflicting findings are likely to be due to the different methods of staining and counting seminal leucocytes, as well as different selection criteria for men representing fertile or infertile groups. These results may also be due to the heterogenous actiology of leucocytospermia (such as sexual abstinence, inflammation, auto immunity etc). Analysis of the heterogenous aetiology of the leucocyte populations in human semen is useful in the diagnosis and therapeutic monitoring of male genital tract infections and of sexually transmitted diseases or autoimmune disease. Analysis of leucocytes is often difficult due to the low percentage of leucocytes in semen, the high number of spermatozoa, and the presence of immature sperm cells, often not easily recognizable from the leucocytes because of their similar staining and cytological characteristics. Whether these cells in semen originate from the reproductive tract (testis, epididymis, and vas) and/or the accessory glands will be important to know. The lack of the clinical significance of leucocytospermia reported in some studies is possibly a reflection of the powerful antioxidant properties of the seminal plasma, which may provide protection against leucocyte-mediated oxidative stress (Aitken et al.1994).

Most of the previously performed studies investigated the effects of these products on sperm motility.

Different hypotheses have been explored recently to explain the relationship between leucocytospermia and sperm structural damage. The hypotheses that sperm structural damage occurs during spermatogenesis is consistent with the findings of a study which showed that infertile men with and without leucocytospermia had similar sperm concentrations in semen (Aziz et al. 2004). Low sperm concentrations would have been expected if the leucocyte induced damage occurred during spermiogenesis (proliferative phase). The mechanism through which leucocytospermia may induce the alteration in sperm structure is not clear. However, one potential explanation is that leucocytospermia could be a marker for an inflammatory process in the testis and in most cases would be related to a subclinical inflammatory process and not due to an overt epididymoorchitis. The presence of proinflammatory mediators such as cytokines in the testis could lead to alterations in the regulation of spermiogenesis by interfering with Sertoli cell function leading to abnormal spermiogenesis (Cohen PE & Pollard JW 1995).

The main objective of this study was to try and resolve an important aspect in clinical andrological examination by determining the relationship of a repertoire of leucocyte subpopulations in the seminal plasma of different subgroups of subfertile men attending a regional andrology clinic. The second objective was to identify the presence if any of leucocytes in seminal plasma of azoospermic men.

MATERIAL & METHODS:

Study Design

A prospective cohort study was designed in which semen was collected from men attending the Reproductive Medicine Unit of the Liverpool Women's Hospital for infertility investigation. The median age of men was 34.5 (range 22 to 49) years. The medium duration of infertility was 5 (range 2 to13) years. 38 of the men consumed alcohol on an average of 10-12 units / week. 20 of the men also smoked on an average of 10-20 cigarettes per day. Men were divided into the different subgroups (normospermic, asthenospermic, oligospermic, oligoasthenospermic, obstructive azoospermic and non obstructive azoospermia) according to the results of the seminal analysis on 2 consecutive semen samples. The seminology laboratory at the hospital carried out standard sperm function tests on each sample, including details on sperm number, motility, and the presence of bound anti-sperm antibodies etc, as detailed by WHO. These men had no other illnesses nor did they take any medication that could have affected their leucocyte subpopulations.

Ethical approval was granted by the Liverpool Research Ethics Committee. Written, informed consent was obtained from 76 men attending the Reproductive Medicine Unit, Liverpool Women's Hospital. Clinical details of both partners were obtained from the case notes.

Semen Collection

Semen was obtained by masturbation after 3- 5 days of sexual abstinence. The samples were collected in a sterile, wide mouthed, non toxic container and processed in the laboratory within 1 hour of ejaculation. Samples did not get exposed to extremes of temperature. They did not come in contact with either lubricants or latex products. All the samples underwent semen analysis in the laboratory in accordance with the 1999 WHO laboratory manual for the

examination of human semen and the study was done using these reference values(1999). Men were divided into the different sub groups according to the results of 2 consecutive semen analysis 6 weeks apart. Given the wide variability between ejaculates, 2 samples were taken to confidently define an individual's phenotype. Asthenospermia was defined as a sperm motility of <40%. Oligospermia was defined as a sperm count <20x10^6. Severe Oligospermia was defined as sperm count <10x10^6. Oligoasthenospermia was defined as a sperm count of 10-20x10^6 and motility of <40%. Azoospermia was defined as complete absence of sperm in the semen either due to germ cell failure such as maturation arrest, hypospermatogenesis or sertoli cell only syndrome (non obstructive group) or due to obstruction in the sperm delivery system. The obstructive azoospermic group had all undergone vasectomy. Although testicular biopsy is the definitive test to rule out the non obstructive from the obstructive groups, the presence or absence of active spermatogenesis can be accurately predicted by measuring the testis volume and serum FSH (Matsumiya et al. 1994). Hence the differentiation between germ cell failure azoospermia and obstructive azoospermia was made by the serum follicle stimulating hormone (FSH) levels, karyotyping and testicular volume. The testicular volume in our non obstructive group was reduced (< 7 ml) and the FSH levels were greater then four times the normal. After semen analysis the remaining sample was processed for the measurement of leucocyte subpopulations. The fertile controls had a normal semen analysis and were ASA and infection negative. This fertile group of men was from the IVF study group and was attending the reproductive medicine unit for purely female factor infertility. Female factor infertility can be divided into four categories: ovarian, tubal, pelvic and uterine. The most common cause of female infertility of ovarian origin is anovulation. Tubal infertility could be caused by infection such as Chlamydia, gonococcal infection and tuberculosis, which damage the fallopian tubes. Previous pelvic or abdominal surgery may distort the anatomy of the tubes. There is a possibility that tubal problems may also be congenital, leading to an

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incomplete development of the fallopian tube. Adhesions that form in the pelvis as a result of an infection or surgery, cause organs or tissues that are normally separate to stick together leading to pelvic infertility. Endometrial polyps and fibroids, if large, may interfere with fertility and cause uterine infertility. Thus isolated conditions of the female are responsible for infertility in 35% of the cases. All the samples underwent testing for genito-urinary infections such as Ureaplasma, Mycoplasma, Chlamydia, Trichomonas Vaginalis, E.Coli and other organisms such as Enterococcus, Streptococcus etc. The swabs were taken directly from semen and sent to the microbiology laboratory for culture to rule out infection.

Preparation of Seminal leucocytes

After semen analysis the remainder of the sample underwent immunocytochemical staining, the established method for accurately detecting leucocyte subpopulations(Wolff 1995). The monoclonal antibodies used were CD2, CD3, CD4 (T helper cells), CD8 (T cytotoxic cells), CD14, CD16 (Granulocytes), CD20 (B cells), CD 45 (Pan Leucocyte), CD56 (Natural killer cells), CD69 (activated T and B cells) and L243.

Semen samples were diluted to 10ml with phosphate buffered saline (PBS), and washed by centrifugation for 10 minutes at 300 g at room temperature. The supernatant was discarded. The final cellular pellet was re-suspended in 2ml of PBS/BSA (bovine serum albumin) {containing 0.1% BSA}. An aliquot of this suspension was mixed 1:1 in Trypan blue (which stains non-viable cells). Using a haemocytometer, the cellular concentration and percentage viability was assessed. By altering the dilution factor of the suspension, the total round cell concentration (including non-viable cells) was 1 x 10^6 / ml in PBS/BSA. The round cells were leucocytes and germ cells. Constantly mixing to ensure an even suspension of cells, 50*ul* was applied to each spot on Teflon-coated slides. Slides were air dried overnight, before fixing for 10 minutes in acetone. Wrapped in foil, slides were stored at -20^0 C until staining.

Slides were removed from the freezer and allowed to reach room temperature. Areas to be stained were marked with a Dako pen to separate adjacent wells. The primary monoclonal antibodies were used as per manufacturer's instructions. Finally, the slides were washed thoroughly in tap water and mounted in Aquamount (BDH. Poole, UK). The number of positively stained (red-pink) leucocytes for each antibody were counted in ten high power fields (HPF = x 320 magnification) using a light microscope fitted with a graticule. Mean values of the ten counts were calculated, and used for an average value per HPF. CD45 binds to a common leucocyte antigen, thus staining all leucocytes. In wells incubated with CD45, both positive and negative (unstained, identified by blue nucleus) cells were counted. This proportion was used to determine the proportion of leucocytes relative to other round cells (immature Germ cells) in the ejaculate. The percentage of CD45 positive cells were determined from the number of CD45 positive cells divided by the sum of both the CD45 positive and negative cells. Since all samples were initially diluted to 1x 10^6/ ml, these results represent total number of leucocytes per ejaculate. The absolute counts of the monoclonal antibodies were used for analysis as some of the monoclonal antibodies can cross react with other cells and hence the medians were used for the analysis rather than expressing them as a percentage of the total round cell count. The specificity of each antibody (the type of leucocyte it binds to, resulting in red-pink staining) was verified on testing the clones obtained from the relevant manufacturers.

Statistical analysis

Statistical analysis was performed using the statistical package for social sciences (SPSSv11) software. The Shapiro Wilkes test was used to test for normal distribution. As our data was not normally distributed, statistical analysis was performed using the Mann- Whitney U test to analyse the difference between cell counts from various groups of patients and sperm parameters. Spearman's rank correlation coefficient was used to determine the correlation

between leucocyte count and sperm parameters. Statistical significance was taken at the 5% confidence level ($p<0.05$). The number of patients is relatively small, as this was aimed to be a pilot study and if significant levels of leucocyte subpopulations were found in the various subgroups then an adequately powered study will be required to determine both the statistical and the clinical significance of these findings.

RESULTS

The CD 45 count in the oligospermic group when compared to the normospermic was not significantly increased as seen in Table 1. Only CD3 was significant ($p<0.003$) as shown in Table 2. CD3 was significant in the asthenospermic ($p<0.001$) group. The major histocompatibility complex (MHC) class 2 cells (L243) were found in all 3 groups. The predominant leucocyte sub group was the granulocytes in the normospermic, oligospermic and the asthenospermic groups. The CD3 (Fig 1) and the CD20 (Fig 2) levels were significantly increased in the asthenospermic group ($p<0.01$ & $p<0.02$) respectively when compared to the normospermic group as shown in Table 2. The T and B cells are significantly increased in the oligoasthenospermic group.

Significant correlations between the T and the B cells exist particularly in the oligosathenospermic groups (Fig 3). They were CD2 and CD8 ($r=0.79$), CD3 and CD8 ($r=0.85$), CD4 and CD8 ($r=0.79$), CD2 and L243 ($r=0.67$), CD3 and L243 ($r=0.70$), CD4 and L243 ($r=0.73$, $p<0.01$). The CD2 & CD3 correlated with CD20 ($r=0.47$, p value<0.05). The other correlations seen only in the oligoasthenospermic group were between CD14, CD16 and L243 $r=0.82$, and CD20 and L243 ($r=0.71$, $p<0.01$).

Other sperm parameters such as morphology were also studied to find out if leucocytospermia had an effect and this is represented in Table 3.

In obstructive azoospermia, there was an increase in the number of T cells (CD3; p< 0.01), B cells (CD 20; p< 0.02), large granular lymphocytes (CD56; p< 0.03) and activated T and B cells (CD69; P<0.03) when compared to the normospermic group. These increased numbers were statistically significant (Table 2). In non obstructive group, there was an increase in the number of T cells (CD3; P= 0.05). Positive correlation was identified between the leucocytes in the non obstructive azoospermic group as shown in Fig 4.

DISCUSSION:

The correlation between leucocytospermia and male infertility is controversial: some studies have even failed to find any association (Fedder, Askjaer, & Hjort 1993) (Curi et al. 2003). However, our study shows different leucocyte species infiltrate the human ejaculate to varying extent and with significant correlations.

Our study, is in agreement with other studies (Aitken et al. 1992) (Bassol, Recio, & de la Cruz 1990) in that there were no statistically significant difference in the CD45 count between the oligospermic and the normospermic groups. Our study also showed for the first time that significant levels of CD3 T lymphocytes were present in the oligospermic group when compared to the normospermic group. This would suggest an increase in the helper T cell activity in this group. There was no increase in the CD14 and CD16 cells in the oligospermic group when compared to the normospermic group. This suggests that these leucocyte subpopulations are not a major factor in influencing sperm structure.

The CD3 were significantly increased in the asthenospermic group when compared to the normospermic group which shows an increase in the helper T cell activity in this group too. CD20 levels were significantly increased in the asthenospermic group when compared to the normospermic group. This raises the possibility that an increase in this cell group could affect sperm motility through an increased ASA production. This ASA could bind to the sperm and decrease its

motility. The activation of a resting helper T cell causes it to release cytokines in particular interferon (IFN) which is known to affect sperm motility. Similarly, activation of resting T helper cells could stimulate the activity of B cells (CD20), the latter producing antibodies and thus further decreasing sperm motility. This is the first study which reports raised T cells (CD 3) and B cells (CD 20) in the asthenospermic group.

Significantly elevated levels of T cells (CD 3 and CD8), B cells (CD20) and large granular lymphocytes (CD56) were found in the oligoasthenospermic group. Most of the previous studies have looked into the total leucocyte count and various sperm parameters. This is the first study that has identified leucocyte sub-type correlations and various sperm parameters. Our study highlights that there is a finely balanced interaction between the T cells and the B cells within the seminal plasma particularly in the oligoasthenospermic group. The likely severity of the condition will probably depend of the predominance of the cell group, as the increased T cell activity is more likely to be of a helper in nature, whereas the increased B cells activity might be the cause of decreased motility due to antibody production and the increased CD56 activity could be removing the non functioning sperm.

We studied the seminal population of leucocytes rather than at the level of the epididymis in both the azoospermic groups. Our study the first in humans identified significant levels of helper T cells (CD3) in both our azoospermic groups and there was a no significant increase in the cytotoxic cells (CD8) in either azoospermic groups. However, in direct contrast, a study found that in the epididymis of rats following a vasectomy, there was a decrease in the T helper cells number and an increase in the T cytotoxic cells number (Hooper et al. 1995). CD20, CD 56 and CD69 counts were significantly elevated in the obstructive group. It is thought that T cells are present in increased numbers as they try to suppress the development of

an immune response to the trapped sperm as a result of an obstruction. This increase in the B cell population in the azoospermic group may result in increased levels of antisperm antibody being secreted which could lead to subsequent subfertility even after vasectomy reversal. We don't propose that there is a cause and effect relationship between these cells and azoospermia, but suggest the site of seminal leucocyte production is not necessarily confined to the vas or the epididymis as once thought.

There are a few limitations to our study. The numbers of patients in each group are small. Hence statistical significance may not correlate with clinical significance. In our study we have not excluded patients who smoke and consume alcohol as none of them were heavy smokers (>20 cigarettes / day) {may increase the seminal leucocyte count and thus increase the seminal oxidative stress or decrease in semen concentration(Close, Roberts, & Berger 1990)} nor chronic alcoholics. {heavy alcohol use was associated with an increase in the seminal leucocytes and chronic alcohol consumption (6 units / day for more than 5 days a week) has shown to affect male reproductive hormones and semen quality}(Muthusami & Chinnaswamy 2005).

Although we have endeavoured to classify our patient sub groups in reference to the WHO manual, some conventional authors may not agree with the quoted reference ranges of semen parameters. Some authors may not be agreeing with our selection of markers used to detect leucocyte subpopulations. CD2 is expressed not only on T cells but also on NK cells and B cells. CD 14 is mostly confined to monocytes / macrophages. However, some non immune cells such as choroids plexus cells are also known to express CD14. Although no significant different levels were detected in our study, some authors would have wanted us to test for the expression of CD 14 by the human germ cells. Correlations between CD 14 and L243 may be considered trivial as monocytes/macrophages express MHC II (clone L243). CD 16 is not restricted to granulocytes and some pro-inflammatory monocytes express CD14 and

CD16.Our hypothesis could have been strengthened by performing a few testicular biopsies on some of our patients with impaired sperm parameters for the presence of inflammatory infiltrates.

The measurement of seminal plasma leucocytes during male genital tract inflammation without an associated contribution of cytokines may have little prognostic value in the evaluation of male infertility. Hence seminal cytokine concentrations were also measured in these sub groups (Seshadri et al. 2009). Significantly higher concentrations of IL-6 ($p < 0.05$), was present in the oligospermic, asthenospermic, oligoasthenospermic and both azoospermic groups when compared to the normospermic group. Significantly higher concentrations of IL-8 ($p < 0.05$), was present in the asthenospermic group when compared to the normospermic group. Significantly higher concentrations of IL-10 ($p < 0.05$), was present in the asthenospermic, oligoasthenospermic and azoospermic obstructive group when compared to the normospermic group. Furthermore IL-6, IL-10, TNF-α and IFN-γ were significantly increased in the obstructive azoospermic group when compared to the normospermic group. A significant correlation was found between IL-10 and IL-11 in the oligospermic ($p<0.05$) and obstructive azoospermic ($p<0.01$) groups. A significant correlation ($p<0.01$) was found between IL-10 and IL-11 and between IL-10 and IFN-γ in the oligoasthenospermic group.

IL-6 positively correlated with L243 in the oligospermic group ($r=0.83$, p value< 0.01) and IFN-γ negatively correlated with CD20 in the severe oligospermic group ($r=-0.74$, $p<0.05$). IL-10 positively correlated with CD69 and IFN-γ positively correlated with CD69 and L243 in the asthenospermic group. IFN-γ correlation between the various leucocyte subpopulations in different subferile men could suggest that IFN-γ, could lead to increased sperm membrane lipid peroxidation. ROS (generated by leucocytes) acts synergistically with pro-inflammatory cytokines to exacerbate the destructive environment for the spermatozoa. We were unable to

detect any significant correlations between the various leucocyte subpopulations and the cytokines IL-10, IL-11 & IL-12 in the various subfertile group studied. This would strongly suggest that these cytokines do not influence spermatogenesis. In the abscence of infection we were unable to detect any correlation between IL-8 or TNF- α expression and the leucocyte subpopulations studied.

The significantly higher frequency of sperm morphological defects in infertile men may suggest that leucocytospermia as an independent variable has a detrimental effect on sperm morphology. This damage could be due to both defective Sertoli cell function and disorganized spermiation or due to alteration in the maturation process that the sperm undergo while transient in the epididymis. Thus, leucocytospermia induced sperm damage may commence during spermiogenesis and continues through spermiation and epididymal migration.

Our study has made an attempt to clarify the possible fertility-reducing effect of leucocytes, the relationship between various sperm characteristics particularly sperm morphology and motility. Our study suggests that sperm structural defects in leucocytospermic men may have been acquired at different stages of spermiogenesis. This could be mediated either through cytokines which have an influence on the sertoli cell function or by the peroxidative damage of ROS released by the activated granulocytes. To the best of our knowledge there have been no studies testing this extent of leucocyte subpopulations using monoclonal antibodies in the different subsets of male subfertility.

CONCLUSION

This is the first study to have identified significant levels of CD3 T lymphocytes and B cells in the various subfertile groups. Our findings suggest that leucocytospermia could impair sperm function through enhanced T helper cell modulation. An increase in the B cell population in the groups may result in increased levels of antisperm antibody being secreted which could impair sperm numbers and function which would result in subfertility. The presence of various leucocyte subpopulations in the obstructive azoospermic group suggests that the site of seminal leucocyte production is not necessarily confined to the vas or the epididymis as previously thought. Natural killer cells could be responsible for mediating sperm damage especially in the oligoasthenospermic group. The presence of increased level of lymphocytes in the reproductive tract could be as a result of an immunological reaction.

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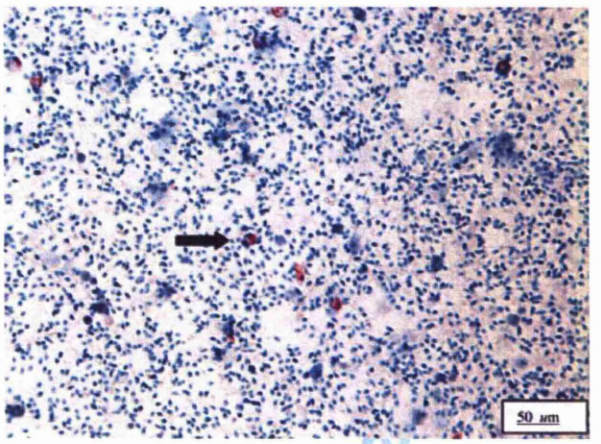

Fig 1: CD 3 cells (stained pink) in the Asthenospermic sample

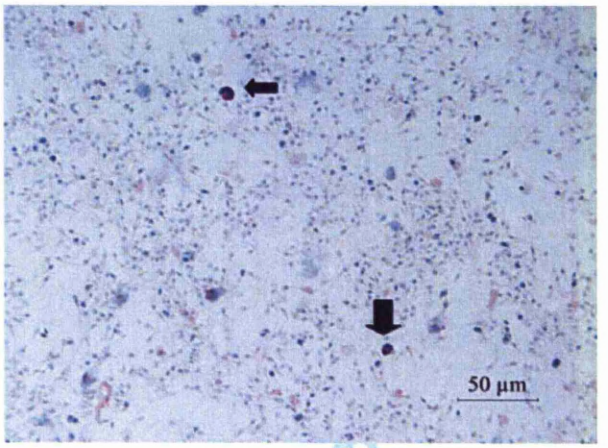

Fig 2: CD 20 cells in the Asthenospermic sample

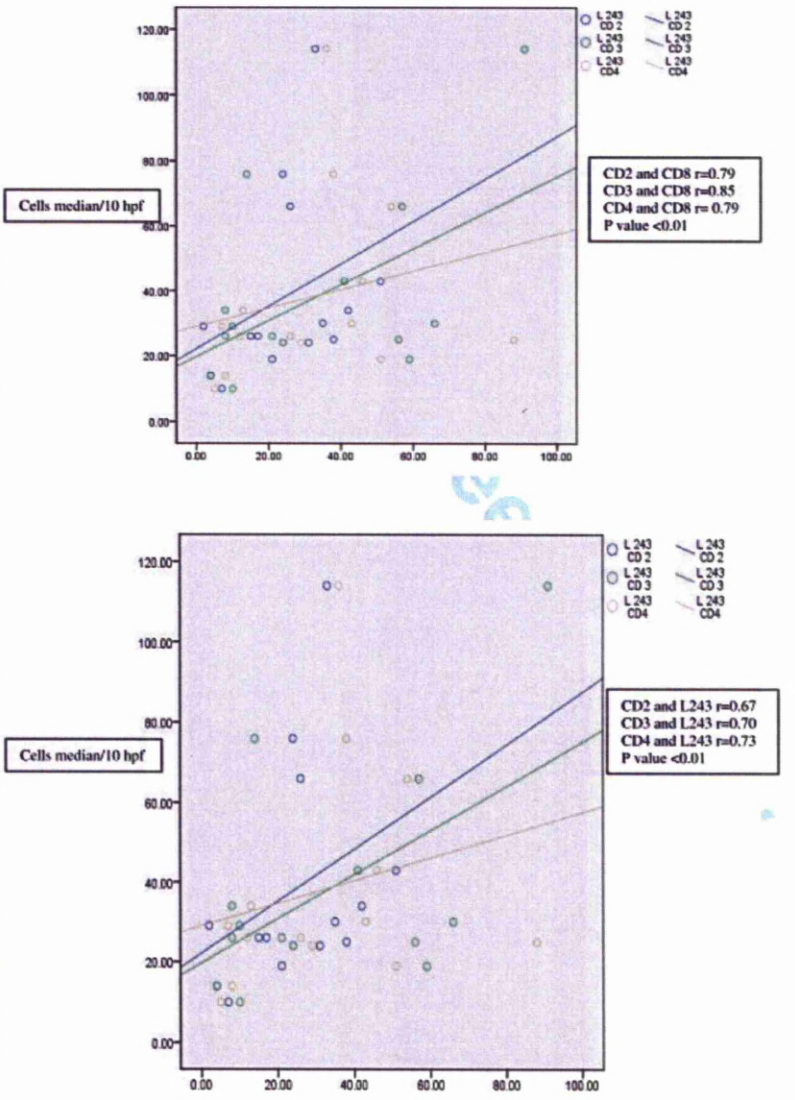

Fig 3: Correlation between the Leucocytes in the Oligoasthenospermic group

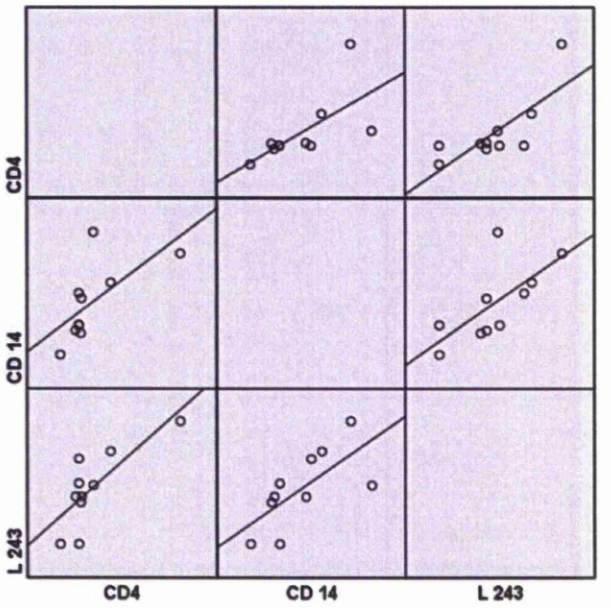

Fig 4: Correlation matrix showing the positive correlations (CD4 and L243 r=0.90, CD14 and CD4 r=0.86, CD14 and L243 r=0.97, p values <0.01) between the leucocytes in the non obstructive azoospermic group.

Table 1: Median and ranges of the Leucocytes (expressed as cells per 10 hpf) in all our various study groups.

	Normospermic N=14 Median / Ranges	Oligospermic N=13 Median / Ranges	Asthenospermic N=10 Median / Ranges	Oligoasthenospermic N=19 Median / Ranges	Azoospermic Obstructive N=10 Median / Ranges	Azoospermic Non Obstructive N=10 Median / Ranges
CD 45%	63 (55.5-76)	76(66-89)	76.7 (69-79)	79 (66-88)	93 (83-97)	80 (64-96)
CD 45	5.9 (3.9-16.7)	9.5 (4.5-13.5)	12.3 (9.6-18.4)	7.5 (4-12.4)	6.65 (3.6-8.4)	5.35 (2.6-11.1)
CD 2	0.7 (0.3-1.9)	1.6(1.1-2.2)	1.6 (1-4)	1.9 (1-2.9)	1.3 (0.7-2.5)	0.9 (0.5-1.5)
CD 3	0.5 (0.3-1.3)	1.7(1.2-2.2)	2.35 (0.9-5)	1.6 (0.9-5.5)	1.35 (1-2.8)	0.85 (0.6-2.5)
CD 4	1 (0.6-2.2)	1.5(1.1-2)	2.15 (1-3)	2 (0.8-3.2)	2.05 (1.2-3)	1.65 (1.5-3.9)
CD 8	0.6 (0.2-1.1)	0.7 (0.4-0.9)	0.95 (0.9-3.2)	1 (0.7-2.6)	1 (0.5-1.7)	0.35 (0.3-2.1)
CD 14	1.2 (0.5-2.8)	1.5 (0.7-1.9)	3.25 (1.8-4.5)	2.1 (0.8-4.7)	2.15 (1.5-3.5)	1.05 (0.7-2.5)
CD 16	2.5 (1.5-5.7)	2.6 (0.7-5.6)	3.85 (1.6-5.5)	2.5 (1.2-7.3)	2.8 (2.2-3.3)	1.7 (1.4-3)
CD 20	0.4 (0.2-1)	0.8 (0.3-1.1)	1.85 (1-5.4)	1 (0.4-2.4)	1.45 (0.7-3.3)	0.6 (0.3-1.1)
CD 56	0.4 (0.3-0.5)	0.6 (0.2-0.9)	0.4 (0.1-0.8)	0.6 (0.3-1.9)	0.8 (0.5-1.4)	0.5 (0.1-3.3)
CD 69	0.5 (0.2-1.2)	1.2 (0.4-1.8)	1.75 (0.7-3.1)	1.1 (0.5-2.3)	1.6 (1-2.4)	0.65 (0.4-1.6)
L243	2.4 (1.7-5.5)	2.5 (1.7-2.9)	2.8 (1.7-6.4)	2.2 (1.6-4.4)	2.5 (1.7-5)	2 (1.3-6.1)

Table 2: P values of the different leucocyte subpopulations from the various subfertile groups when compared to the normospermic group (*significance at 5% level, **significance at the 1% level)

	Normospermic vs Oligospermic P value	Normospermic vs Asthenospermic P value	Normospermic vs Oligoasthenospermic P value	Normospermic vs Azoospermic Obstructive P value	Normospermic vs Azoospermic Germ cell failure P value
CD 45%	0.43	0.14	0.07	0.001**	0.1
CD 45	0.69	0.39	0.945	0.82	0.47
CD 2	0.21	0.15	0.09	0.45	0.72
CD 3	**0.003****	**0.001***	**0.004****	**0.01***	**0.05***
CD 4	0.34	0.19	0.2	0.22	0.17
CD 8	0.39	0.06	**0.02***	0.1	0.82
CD 14	0.87	0.08	0.22	0.11	0.72
CD 16	0.71	0.82	0.94	0.96	0.44
CD 20	0.47	**0.02***	**0.02***	**0.02***	0.33
CD 56	0.52	0.8	**0.05***	**0.03***	0.47
CD 69	0.18	0.07	0.1	**0.03***	0.45
L243	0.80	0.93	0.6	0.96	0.68

Table 3: Median and ranges of the sperm parameters in the various subfertile groups are shown in comparison to the normospermic group.

Sperm Parameters	Normospermic (n=14)	Oligospermic (n=13)	Asthenospermic (n=10)	Oligoasthenospermic (n=19)
Sperm count 10^6/ml	43 (28-75)	10 (5-11)	46.5 (34-83)	5 (2-15)
Motility %	25 (20-40)	27.5 (25-30)	13.75 (2.5-17.5)	5 (2.5-15)
Morphology % normal forms	28 (18-32)	18 (12-22)	10 (9-12)	11 (8-16)